Wpływ pestycydów na pszczołę murarkę *Osmia bicornis* w krajobrazie rolniczym

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Effect of pesticides on the red mason bee *Osmia bicornis*
in the agricultural landscape

Anna Misiewicz

Rozprawa doktorska

Instytut Ochrony Przyrody Polskiej Akademii Nauk

Kraków, 2024

AUTOR: **MGR ANNA MISIEWICZ**
Instytut Ochrony Przyrody Polskiej Akademii Nauk
al. A. Mickiewicza 33, 31-120 Kraków

PROMOTOR: **DR HAB. AGNIESZKA BEDNARSKA, PROF. IOP PAN**
Instytut Ochrony Przyrody Polskiej Akademii Nauk
al. A. Mickiewicza 33, 31-120 Kraków

PROMOTOR POMOCNICZY: **PROF. DR HAB. RYSZARD LASKOWSKI**
Instytut Nauk o Środowisku,
Uniwersytet Jagielloński
ul. Gronostajowa 7, 30-387 Kraków

SPIS TREŚCI

SPIS PRAC

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II. Misiewicz, A., Mikołajczyk, Ł., Bednarska, A. J., 2023. Floral resources, energetic value and pesticide residues in provisions collected by *Osmia bicornis* along a gradient of oilseed rape coverage. Scientific Reports 13, 13372. https://doi.org/10.1038/s41598-023-39950-5 (IF = 4,6; 140 pkt. MNiSW).

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IV. Misiewicz, A. Filipiak, Z. M., Kadyrova, K., Bednarska, A. J., 2024. Combined effects of three insecticides with different modes of action on biochemical responses of the solitary bee *Osmia bicornis*. Chemosphere, 142233. https://doi.org/10.1016/j.chemosphere.2024.142233 (IF = 8.8; 140 pkt. MNiSW).

FINANSOWANIE BADAŃ

Badania wchodzące w skład rozprawy doktorskiej zostały sfinansowane przez Narodowe Centrum Nauki w ramach projektu SONATA (UMO-2017/26/D/NZ8/00606): „Wpływ pestycydów na pszczołę murarkę *Osmia bicornis* w krajobrazie rolniczym: toksyczność mieszanin i ewolucja odporności" (kierownik: dr hab. Agnieszka Bednarska). Rozszerzenie badań wchodzących w skład artykułu trzeciego było możliwe dzięki finansowemu wsparciu otrzymanemu z programu badań i innowacji Unii Europejskiej Horyzont 2020 w ramach projektu EcoStack („Stacking of ecosystem services: mechanisms and interactions for optimal crop protection, pollination enhancement, and productivity", nr 773554) realizowanego w Instytucie Nauk o Środowisku Uniwersytetu Jagiellońskiego.

PODZIĘKOWANIA

Składam serdeczne podziękowania mojej promotor, dr hab. Agnieszce Bednarskiej, prof. IOP PAN, za nieocenioną pomoc podczas całego doktoratu, bez której powstanie poniższej rozprawy nie byłoby możliwe. Dziękuję za możliwość uczestniczenia w projekcie, za przekazaną wiedzę, za konstruktywną krytykę oraz wsparcie.

Chciałabym również podziękować mojemu promotorowi pomocniczemu, prof. dr. hab. Ryszardowi Laskowskiemu, za cenne wskazówki oraz pomoc w rozwiązywaniu problemów naukowych, które napotkałam w trakcie swoich badań.

Dziękuję koleżankom i kolegom z Instytutu Ochrony Przyrody PAN za miłe towarzystwo w okresie przygotowywania rozprawy doktorskiej, a szczególnie dr Dorocie Kotowskiej i dr Zuzannie Filipiak za wsparcie oraz udzielanie cennych wskazówek.

Dziękuję także całemu Zespołowi Ekosystemów Lądowych i Ekotoksykologii INoŚ UJ za towarzystwo, pomoc podczas badań, oraz za możliwość korzystania z laboratoriów.

Szczególne podziękowania kieruję do mojego partnera, za motywowanie mnie do działania, wsparcie emocjonalne i nieustającą cierpliwość oraz rodzicom za umożliwienie realizacji wszystkich moich pomysłów.

Na koniec pragnę podziękować mojemu czworonożnemu przyjacielowi za towarzyszenie mi podczas całego doktoratu oraz za dbanie o moje zdrowie fizyczne i psychiczne dzięki codziennym spacerom.

STRESZCZENIE

Rosnące zapotrzebowanie na żywność i chęć maksymalizacji zysków z upraw, napędzane wzrostem populacji ludzkiej, negatywnie wpływają na bioróżnorodność owadów, w tym zapylaczy, których rola w produkcji rolnej jest nie do przecenienia. Obserwowany w ostatnich latach gwałtowny spadek liczebności zapylaczy, przede wszystkim w krajobrazie rolniczym, nie tylko negatywnie wpływa na bioróżnorodność, ale również może zaszkodzić gospodarce rolnej i doprowadzić do wzrostu cen żywności. Dlatego ważne jest, aby utrzymać wystarczająco liczebne populacje owadów zapylających na terenach rolniczych. To z kolei wymaga utrzymania niezbędnych warunków siedliskowych, zapobiegających ekstynkcji zapylaczy. Intensyfikacja rolnictwa poprzez zwiększanie areału upraw i stosowanie pestycydów, zwłaszcza insektycydów, zagraża zapylaczom. Wiele upraw, takich jak zboża, zwłaszcza w wielkoobszarowych monokulturach, wokół których brakuje miedz, zadrzewień i innych ostoi dla zapylaczy, jest nieatrakcyjne dla owadów zapylających i nawet bez udziału pestycydów może znacząco wpływać na ich bioróżnorodność. Z drugiej strony, rośliny masowo kwitnące, np. rzepak, mogą dostarczać nektar i pyłek dzikim zapylaczom. Jednak nawet w uprawach atrakcyjnych dla zapylaczy, o dużych zasobach pokarmowych, stosuje się pestycydy. Dlatego badania nad łącznym wpływem struktury krajobrazu i pestycydów na pszczoły, których przedstawicielem jest murarka ogrodowa (*Osmia bicornis*), są niezbędne, aby w pełni zrozumieć ich skutki dla kolejnych pokoleń tych pszczół i wprowadzić odpowiednie strategie ochrony zapylaczy. Presja powodowana przez intensyfikację rolnictwa może ujawniać się w postaci bezpośrednich efektów widocznych w przeżywalności osobników rodzicielskich, ale niekorzystne warunki życia rodziców mogą też wpływać na rozwój ich potomstwa (ang. *carry-over effect*), czy też ujawniać się dopiero w następnym pokoleniu w postaci efektów matczynych (ang. *maternal effect*). Dlatego, jednym z głównych celów niniejszej rozprawy było zbadanie, czy intensyfikacja rolnictwa, wyrażona różną strukturą krajobrazu rolniczego z gradientem udziału upraw rzepaku wokół gniazda, a tym samym wzrastającą presją ze strony rolnictwa, wpływa negatywnie na parametry populacyjne pszczoły samotnej *O. bicornis* i jej wrażliwość na przedstawicieli głównych grup stosowanych obecnie insektycydów oraz czy konsekwencje rozwoju osobników w krajobrazie rolniczym zdominowanym przez rzepak są widoczne tylko na kolejnych

etapach rozwoju w danym pokoleniu czy też również w pokoleniu kolejnym, rozwijającym się już w warunkach pozbawionych presji rolnictwa (artykuł I). Ponadto sprawdzano czy wraz ze wzrostem udziału rzepaku wokół gniazd pszczół *O. bicornis* maleje różnorodność pyłku i wzrasta stężenie pestycydów w pyłku (artykuł II).

Częstym zabiegiem stosowanym w rolnictwie jest używanie mieszanin dwóch lub więcej różnych pestycydów, na ogół należących do różnych grup chemicznych. Skutki działania pestycydów w mieszaninie mogą okazać się znacznie bardziej szkodliwe dla organizmu niż suma działania pojedynczych substancji, co wynika z możliwości synergistycznego działania dwóch (lub więcej) substancji na organizm. Działanie kilku substancji może być jednak też antagonistyczne, czyli powodować skutki słabsze niż wynikałoby to z prostego sumowania się efektów działania tych substancji stosowanych niezależnie. Ponadto, zapylacze często narażone są na subletalne dawki substancji chemicznych, tj. takie, które nie powodują natychmiastowych i jednoznacznych skutków toksycznych. Sprawdzenie wpływu mieszanin insektycydów należących do różnych grup na przeżywalność (artykuł III) oraz na wybrane parametry biochemiczne, tj. aktywność trzech enzymów (acetylocholinoesterazy (AChE), S-transferazy glutationowej (GST) i esterazy (EST)) oraz poziom ATP (artykuł IV) to kolejne cele, jakie postawiono w niniejszej rozprawie.

Przeprowadzone badania wykazały, że rozwój larwalny w warunkach monokultury rzepaku (tj. w obszarach ze wzrastającym udziałem upraw rzepaku wokół gniazda pokolenia rodzicielskiego (P)) wpłynął negatywnie na niektóre parametry populacyjne potomstwa (F1), tj. obniżał sukces wylęgania pszczół z kokonów i zwiększał wrażliwość samic na insektycyd Dursban 480 EC, ale efekty te w większości zanikły w następnym pokoleniu (F2). W pokoleniu F2, rozwijającym się w terenie pozbawionym presji rolniczej (łąki śródleśne), wpływ udziału rzepaku i struktury krajobrazu wokół gniazda założonego przez samice z pokolenia P był widoczny tylko w zaburzonej proporcji płci, tj. w większym udziale samic. Ponadto wykazano, że obecność naturalnych i półnaturalnych elementów krajobrazu ma istotne znaczenie dla rozwoju pszczół samotnych w krajobrazie rolniczym. Udział rzepaku wokół gniazd pszczół nie wpływał na różnorodność florystyczną pyłku, wartość energetyczną czy poziom skażenia pyłku zbieranego przez samice z pokolenia P dla swojego potomstwa F1, ale różnorodność i

wartość energetyczna tego pyłku, zależały od obecności innych niż rzepak elementów krajobrazu. Ponadto, różnorodność pyłku malała, a wartość energetyczna rosła wraz z różnorodnością krajobrazu. W badanym krajobrazie rolniczym, pszczoły zebrały pyłek z 28 taksonów roślin, z dominacją *Brassica napus*, *Quercus* sp., *Ranunculus* sp., Poaceae i *Acer* sp. W pyłku wykryto pozostałości 12 pestycydów, a acetamipryd, azoksystrobina, boskalid i dimetoat były najczęściej wykrywane. Skażenie pyłku pestycydami malało wraz ze wzrostem jego różnorodności florystycznej.

Wbrew oczekiwaniom, badania laboratoryjne na dorosłych samicach *O. bicornis* wskazały albo brak interakcji (w mieszaninie Sherpa 100 EC × Dursban 480 EC) albo antagonistyczne działanie badanych mieszanin insektycydów na przeżywalność *O. bicornis*. Interakcje antagonistyczne wystąpiły w mieszaninach insektycydów, w których jeden należał do pyretroidów (Sherpa 100 EC lub Karate Zeon 050 CS), a drugi był neonikotynoidem (Mospilan 20 SP) lub sulfoksyminem (Closer). Pyretroid Sherpa 100 EC wpływał na wszystkie badane biomarkery (AChE, GST, EST, ATP), a fosforoorganiczny Dursban 480 EC na aktywność AChE i EST oraz poziom ATP. Złożone interakcje pomiędzy tymi insektycydami oraz neonikotynoidem (Mospilan 20 SP) wpływały na poziom ATP, dając wyniki, których nie dałoby się przewidzieć testując każdy z insektycydów osobno.

Uzyskane wyniki pokazują, że rozwój larwalny w warunkach dominacji upraw rzepaku negatywnie wpływa na niektóre parametry historii życiowej pszczół, ale efekty te w większości zanikają w kolejnym pokoleniu. Daje to nadzieję na szybką odbudowę populacji dzikich pszczół, o ile zapewni się ku temu dogodne warunki. Obecność takich elementów krajobrazu jak zbiorniki wodne wraz z otaczającą je roślinnością, łąki, lasy oraz struktura krajobrazu charakteryzująca się dużą długością granic między polami a siedliskami naturalnymi, powinny być uwzględnione w ochronie owadów pożytecznych w krajobrazach rolniczych, podobnie jak zapewnienie różnorodnej bazy pokarmowej. Wykazano, że stosowanie pyretroidu w mieszaninie z neonikotynoidem lub sulfoksaminem może być bezpieczniejsze dla *O. bicornis* niż stosowanie tych insektycydów pojedynczo, a wyniki z analiz poziomu ATP dodatkowo sugerują, że insektycydy fosforoorganiczne nie powinny być mieszane z neonikotynoidami i/lub

pyretroidami, ponieważ takie kombinacje insektycydów negatywnie wpływają na metabolizm pszczół samotnych.

SUMMARY

The growing demand for food and the desire to maximize crop profits, driven by human population growth, negatively affect the biodiversity of insects, including pollinators, whose role in agricultural production cannot be overestimated. The strong decline in the number of pollinating insects observed in recent years, mainly in agricultural landscapes, not only negatively affects biodiversity but can also harm the agricultural economy and lead to higher food prices. Therefore, it is important to maintain a sufficiently large population of pollinating insects in agricultural areas. This, in turn, requires maintaining the necessary habitat conditions to prevent the extinction of pollinators. The intensification of agriculture through increasing crop acreage and the use of pesticides, especially insecticides, threatens pollinators. Many crops, such as cereals, especially in large monocultures that lack baulks, trees, and other refuges for pollinators, are unattractive to pollinators and even without pesticides can significantly affect their biodiversity. On the other hand, mass-flowering plants, such as oilseed rape, can provide nectar and pollen to wild pollinators. However, even crops attractive to pollinators, which offer abundant food supply, are treated with pesticides. Therefore, research on the combined effects of landscape structure and pesticides on bees, with the red mason bee (*Osmia bicornis*) as a representative, is essential to fully understand their effects on future generations of these bees and to implement adequate conservation strategies for pollinators. Pressure from agricultural intensification can manifest itself as direct effects visible in the survival rate of parental individuals, but unfavourable living conditions of parents can affect the development of their offspring (*carry-over effect*) or reveal themselves in the next generation as maternal effects. Therefore, one of the main objectives of this dissertation was to investigate whether agricultural intensification, expressed in the structure of agricultural landscape with a gradient of the share of oilseed rape around the nest, and thus increasing pressure from agriculture, negatively affects population parameters of the solitary bee *O. bicornis* and its sensitivity to the representatives of the major groups of insecticides currently in use, and whether the consequences of the development of individuals in the landscape dominated by oilseed rape are visible only at subsequent developmental stages in the given generation, or also in the next generation developing under conditions without agricultural pressure (article I). Additionally, it was examined whether pollen diversity decreases and pollen pesticide contamination increases with the increase in the proportion of oilseed rape around the nests of *O. bicornis* bees (article II).

It is common practice in agriculture to use mixtures of two or more different pesticides, usually belonging to different groups. The effects of pesticides in a mixture may be much more harmful than the sum of the effects of individual substances, due to possible synergistic effects of two (or more) substances on the organism. However, the effect of several substances can also be antagonistic, i.e., weaker than expected from a simple summation of the effects of these substances used independently. In addition, pollinators are often exposed to sublethal doses of chemicals, i.e., doses that do not cause immediate and unambiguous toxic effects. Studying effects of a mixture of insecticides belonging to different groups on survival (article III) and on selected biochemical parameters, i.e., the activity of three enzymes (acetylcholinesterase (AChE), S-glutathione transferase (GST), and esterase (EST)), and the level of ATP (article IV), were other goals of this dissertation.

The study showed that larval development in areas dominated by oilseed rape monoculture (i.e., with the elevated share of oilseed rape around the nest of the parental generation (P)) negatively affected some population parameters of offspring (F1), namely decreased the emergence success of bees from cocoons and increased the sensitivity of females to the insecticide Dursban 480 EC, but these effects have mostly disappeared in the next generation (F2). In the F2 generation that developed in an area without agricultural pressure (mid-forest meadows), the effect of the share of oilseed rape and the landscape structure around the nest established by the females of the P generation was evident only in the distorted sex ratio, i.e., the higher proportion of females. In addition, the presence of natural and semi-natural landscape elements was shown to be important for the development of bees in agricultural landscapes. The share of oilseed rape around the bees' nests did not affect the floral diversity, energy value or contamination level of pollen collected by P generation females for their F1 offspring, but the diversity and energy value of this pollen depended on the presence of landscape elements other than oilseed rape. Moreover, pollen diversity decreased, and energy value increased with landscape diversity. In the studied agricultural landscape, bees collected pollen from 28 plant taxa, among which *Brassica napus*, *Quercus* sp., *Ranunculus* sp., Poaceae, and *Acer* sp. were predominant. Residues of 12 pesticides were detected in the pollen, with acetamiprid, azoxystrobin, boscalid, and dimethoate being the most frequently detected. Pollen contamination with pesticides decreased with increasing floral diversity.

Contrary to expectations, laboratory tests on adult females showed either no interaction (in the Sherpa 100 EC × Dursban 480 EC mixture) or antagonistic effects of the tested insecticide mixtures on the *O. bicornis* survival. Antagonistic interactions occurred in mixtures in which

one insecticide belonged to pyrethroids (Sherpa 100 EC or Karate Zeon 050 CS), and the other was a neonicotinoid (Mospilan 20 SP) or a sulfoxymine (Closer). The pyrethroid Sherpa 100 EC affected all biomarkers tested (AChE, GST, EST, ATP), and organophosphate Dursban 480 EC affected AChE and EST activity and ATP levels. The complex interactions between these insecticides and the neonicotinoid (Mospilan 20 SP) affected ATP levels, giving results that could not be predicted by testing each insecticide separately.

The results show that larval development under conditions of oilseed rape dominance negatively affects some life history parameters of bees, but these effects mostly disappear in the next generation. This gives hope for the rapid recovery of wild bee populations, as long as favourable conditions are provided. The presence of landscape elements such as water bodies and vegetation close to water, meadows, forests, and a landscape structure with long boundaries between fields and natural habitats should be considered in the protection of beneficial insects in agricultural landscapes, as well as the provision of a diverse food base. It has been shown that using a pyrethroid in a mixture with a neonicotinoid or sulfoximine may be safer for *O. bicornis* than using these insecticides alone. Further, results of ATP level analyses suggest that organophosphate insecticides should not be mixed with neonicotinoids and/or pyrethroids, as such insecticide combinations negatively affect bee metabolism.

WPROWADZENIE - SYNTEZA BADAŃ I WYNIKÓW

Owady zapylające odgrywają istotną rolę w utrzymaniu różnorodności biologicznej, dobrym funkcjonowaniu ekosystemów i bezpieczeństwie żywnościowym, a także mają wiele wartości społeczno-ekonomicznych jako źródło dochodu, inspiracji i wartości kulturowej dla społeczeństwa (Potts i in., 2016). W związku ze wzrastającą liczbą ludności na świecie wzrasta zapotrzebowanie na żywność, w której produkcji duży udział mają zapylacze (Klein i in., 2007). Zapylanie roślin przez owady jest kluczową usługą ekosystemową, niezbędną w większości upraw (IPBES, 2016), i jest wyceniane w skali świata na 153 miliardy dolarów rocznie (Gallai et al., 2009). Jednak w ostatnich latach jest coraz więcej dowodów na spadek liczebności stawonogów, w tym dzikich owadów zapylających, szczególnie w Europie i Ameryce Północnej, gdzie entomofauna była szeroko badana (Hallmann i in., 2017; Koh i in., 2016; Powney i in., 2019; Seibold i in., 2019). Podobne trendy zaobserwowano jednak również w innych regionach świata (Millard i in., 2021). Szczególnie zauważalny jest spadek bogactwa gatunkowego dzikich zapylaczy; przykładowo w Wielkiej Brytanii pomiędzy 1980 a 2013 rokiem liczba gatunków należących do tej grupy zmniejszyła się o 33% (Powney et al., 2019). Może to negatywnie wpłynąć na gospodarkę i skutkować wzrostem cen żywności (Kevan i Phillips, 2001). W związku z tym ważne jest, aby na terenach rolniczych utrzymywać populacje zapylaczy na odpowiednim poziomie. Aby spełnić to założenie, niezbędne jest zapewnienie im odpowiednich warunków siedliskowych. Intensywny rozwój rolnictwa przyczynia się jednak do powstawania wielkoobszarowych monokultur upraw, co prowadzi do spadku heterogeniczność krajobrazu - zanikają miedze, zadrzewienia śródpolne, nieużytki. Takie monokultury często nie są atrakcyjne dla zapylaczy (np. uprawa zboża), ze względu na brak roślin nektarodajnych. Z drugiej strony, rośliny masowo kwitnące mogą dostarczać nektaru i pyłku dzikim zapylaczom i chociaż jest to monotonna i chwilowa baza pokarmowa, to dla niektórych gatunków, takich jak pszczoła samotna *Osmia bicornis*, dla której okres budowy gniazda przypada na okres kwitnienia rzepaku (kwiecień – maj), może to być bardzo istotny element bazy pokarmowej. Jednak także w uprawach roślin potencjalnie atrakcyjnych pokarmowo dla zapylaczy (np. uprawa rzepaku) stosuje się wiele środków ochrony roślin (herbicydy, insektycydy, fungicydy), które mogą negatywnie oddziaływać na zapylacze (Sgolastra i in., 2018). Wszelkie chwasty - potencjalne źródło pożywienia dla zapylaczy, są w takich miejscach systematycznie zwalczane. W środowiskach zdominowanych przez monokultury brakuje różnorodnej bazy pokarmowej, co również może również negatywnie wpływać na populacje zapylaczy, gdyż dzikie zapylacze często potrzebują zróżnicowanych

zasobów kwiatowych, aby uzyskać lepszą wydajność reprodukcyjną (Klaus i in., 2021). Na przykład wykazano, że wysoki udział terenów rolniczych wokół gniazd *Osmia cornifrons* zmniejszył liczbę potomstwa samic poprzez zmniejszenie różnorodności pyłku w diecie pszczół (Centrella i in., 2020).

Mimo, że w ostatnich latach liczba badań nad wpływem cech krajobrazu na różne gatunki pszczół wzrosła (np. Bednarska i in., 2021; Coudrain i in., 2016; Coutinho i in., 2021; Schüepp i in., 2011; Sõber i in., 2020), wciąż niewiele wiadomo o tym, jaki jest łączny wpływ struktury krajobrazu i stosowania pestycydów, czyli ogólna presja ze strony rolnictwa, na cechy historii życiowej dzikich pszczół samotnych. Koszty życia organizmów ponoszone na wcześniejszych etapach rozwoju (np. na etapie rozwoju larwalnego) mogą ujawniać się dopiero na późniejszych etapach życia (np. w stadium dorosłym) (Anderson i Harmon-Threatt, 2019; Stuligross i Williams, 2021), co wiąże się z tzw. „efektem przenoszenia" (ang. *carry-over effect*; O'Connor i in., 2014). „Efekt przenoszenia" może występować między poszczególnymi etapami historii życiowej, etapami rozwojowymi, etapami fizjologicznymi lub etapami społecznymi, z których każdy występować może w odrębnej skali czasowej (O'Connor i in., 2014). Co więcej, środowisko życia rodziców może wpływać na jakość ich późniejszego potomstwa i ujawniać się dopiero w kolejnym pokoleniu (Mousseau i Fox, 1998). To, czy ewentualny wpływ presji rolnictwa jest widoczny tylko w postaci bezpośrednich efektów u osobników dorosłych, czy też ujawnia się na kolejnych etapach rozwoju w danym pokoleniu lub też dopiero w kolejnych pokoleniach, ma istotny wpływ na dobór odpowiednich strategii, jakie powinny być wdrażane w celu zapewnienia ochrony zapylaczy w krajobrazie rolniczym.

Jak wspominano powyżej, nie tylko brak naturalnych lub półnaturalnych elementów struktury krajobrazu, ale również ilość i rodzaj stosowanych pestycydów wpływa na obecność i liczebność zapylaczy w krajobrazie rolniczym. Całkowite zużycie pestycydów w rolnictwie na świecie wyniosło 3,5 Mt substancji czynnych w 2021 r., co stanowi wzrost o 4% w ciągu roku i dwukrotny wzrost od 1990 r. (FAO, 2023). Z punktu widzenia ochrony zapylaczy, największe zagrożenie stanowią środki stosowane do zwalczania szkodników owadzich – insektycydy, gdyż działają one na mechanizmy uniwersalne w świecie zwierząt, związane z funkcjonowaniem układu nerwowego (np. blokują kanały sodowe w komórkach nerwowych oraz Ca2+-ATPazę, hamując w ten sposób repolaryzację neuronów, co uniemożliwia prawidłowe przekazywanie sygnału nerwowego; poprzez działanie na ATPazy, uszkadzają funkcjonowanie mitochondriów; blokują acetylocholinesterazę, uniemożliwiając rozkład acetylocholiny, a tym samym pozostawiając układ nerwowy w stanie wzbudzenia, co

uniemożliwia normalne przewodzenie sygnałów nerwowych; wiążą się z receptorami acetylocholiny na błonie postsynaptycznej upośledzając przekazywanie sygnałów nerwowych pomiędzy neuronami).

Popularnym zabiegiem stosowanym w rolnictwie jest używanie mieszanin dwóch lub więcej pestycydów, często należących do różnych grup chemicznych. Skutki działania pestycydów w mieszaninie mogą okazać się dla organizmu bardziej szkodliwe niż wynikałoby to z sumy skutków pojedynczych substancji. Takie synergistyczne działanie pestycydów na przeżywalność stwierdzono na przykład u *O. bicornis* w przypadku klotianidyny (insektycyd) i propikonazolu (fungicyd) (Sgolastra i in., 2018). Z kolei antagonizm (gdzie toksyczność dwóch lub więcej pestycydów w mieszaninie jest niższa niż suma toksyczności każdego pestycydu zastosowaego osobno) stwierdzono na przykład w badaniach przeżywalności u *A. mellifera* po narażeniu na mieszaninę fungicydów hamujących biosyntezę steroli z niską dawką tau-fluwalinatu (insektycyd) (Johnson i in., 2013). Chociaż problem toksyczności mieszanin był szeroko dyskutowany w ostatnich dwóch dekadach (Van Gestel et al., 2010), zaskakująco mało wiadomo na temat wpływu stosowania mieszanin pestycydów na owady zapylające, szczególnie pszczoły samotne (Tosi i in., 2022). Interakcje między różnymi insektycydami badano tylko w około 6% eksperymentów związanych z pestycydami na pszczołach miodnych (Benuszak i in., 2017). Carnesecchi i in. (2019) wykazali, że spośród 957 publikacji, tylko 14 dotyczyło wpływu mieszanin substancji, przy czym większość (10 artykułów) koncentrowała się na *A. mellifera*, podczas gdy tylko cztery artykuły obejmowały badania na rodzajach *Bombus* i *Osmia*. Wynika to prawdopodobnie z faktu, że obecnie ocena ryzyka ekologicznego nie uwzględnia wpływu interakcji między środkami ochrony roślin, nawet jeśli wiele z nich jest powszechnie stosowanych jako mieszaniny lub w krótkich odstępach czasu. Co więcej, badania nad toksycznością pestycydów często polegają na testowaniu substancji czynnej pestycydu, a nie całego środka ochrony roślin dostępnego komercyjnie, który zawiera zwykle także różnego typu rozpuszczalniki, substancje utrwalające, aktywatory i adiuwanty wzmacniające działanie substancji czynnej (Mullin i in., 2015), które także mogą negatywnie wpływać na pszczoły (Heys i in., 2016). Badania wykazały, że te dodatkowe substancje mogą być toksyczne zarówno dla larw, jak i dorosłych pszczół miodnych (Shannon i in., 2023; Zhu i in., 2014). Z tego powodu ważne jest, aby poznać nie tylko wpływ mieszanin różnych substancji czynnych, ale także wpływ mieszanin różnych środków ochrony roślin na owady zapylające. Ponadto, zapylacze są często narażone na subletalne dawki substancji chemicznych, tj. takie, które nie powodują natychmiastowych i jednoznacznych

skutków toksycznych, ale mogą zaburzać różne procesy fizjologiczne i biochemiczne, co w przypadku pszczół innych niż te z rodzaju *Apis* jest słabo poznane (Lehmann i Camp, 2021). Lepsze zrozumienie zagrożeń związanych z łącznym stosowaniem insektycydów i ich wpływu na fizjologię i metabolizm pszczół samotnych stanowi ważny krok w kierunku ich lepszej ochrony (Leroy i in., 2023, Raine i Rundlöf, 2024).

Ocena potencjalnego wpływu pestycydów na owady zapylające skupia się głównie na badaniu pszczoły miodnej (*Apis mellifera*), która jest standardowym gatunkiem uwzględnianym w testach ekotoksykologicznych, będących podstawą prawnych regulacji stosowania pestycydów w UE. Istnieje jednak rosnąca potrzeba uwzględnienia w ocenie ryzyka ekologicznego (ang. *ecological risk assessment*, ERA) innych gatunków pszczół, o różnej biologii i ekologii (Schmolke i in., 2021; Williams et al., 2023). Tym bardziej, że około 20% usług zapylania w produkcji rolnej jest świadczonych przez dzikie pszczoły (Losey i Vaughan, 2006), w tym pszczoły samotne z rodzaju *Osmia,* które często są bardziej skutecznymi zapylaczami niż pszczoły miodne (Garibaldi et al., 2013). Pszczoły z rodzaju *Osmia* odgrywają ważną rolę w zapylaniu takich gatunków roślin, jak jabłoń, wiśnia czy rzepak (Bosch i Kemp, 2002). Ze względu na różnice (nawet 25-krotne) we wrażliwości na niektóre pestycydy w porównaniu z pszczołami miodnymi i trzmielami (Heard i in., 2017) niedawno zostały rekomendowane jako gatunki modelowe w ocenie ryzyka ekologicznego ze strony pestycydów dla pszczół (EFSA i in., 2023), chociaż standardowe testy OECD dla tych pszczół nie zostały jeszcze zatwierdzone. Ponadto, coraz więcej mówi się o tym, że ocena ryzyka (ERA) powinna odbywać się na poziomie krajobrazu (Topping et al., 2020), co prowadzi do rozwoju nowych narzędzi (Poulsen et al., 2023) pozwalających na ocenę w jaki sposób ryzyko różni się w zależności od krajobrazu i umożliwiających ocenę skutków narażenia na wiele pestycydów (np. mieszanek pestycydów w jednym oprysku lub stosowania serii oprysków w krótkich odcinkach czasu).

Osmia bicornis należy do rodziny Megachilidae i jest szeroko rozpowszechniona w Europie i Azji Zachodniej (Amiet et al., 2004). Cykl życia tego gatunku, podobnie jak innych pszczół samotnych, obejmuje kilka etapów. Wiosną, po wygryzieniu się z kokonów samice są zapładniane przez samce i poszukują miejsca do gniazdowania (preferowane są m.in. puste łodygi roślin czy dziury w drewnie). W kolejnym etapie samica zaopatruje komórkę gniazdową w zapas pyłku i nektaru i składa w niej jajo. Następnie zabezpiecza wejście do komórki wilgotną gliną/ziemią. Jedna samica składa około 30 jaj (Sedivy i Dorn, 2014). Z jaj rozwijają się larwy, które żywią się zapasami nektaru i pyłku. Po około 35.5±1.99 i 31.6±2.41 dniach, odpowiednio dla samic i samców, przedpoczwarki zaczynają tkać kokony (Giejdasz i

Wilkaniec, 2002). Następnie zaczyna się stadium poczwarki, która przekształca się w dorosłego osobnika, który hibernuje w kokonie do wiosny.

CEL BADAŃ

Celem prowadzonych przeze mnie badań było sprawdzenie wpływu struktury krajobrazu rolniczego i toksyczności stosowanych w rolnictwie pestycydów na pszczoły samotne – murarki ogrodowe *Osmia bicornis*. Dzięki połączeniu badań terenowych i laboratoryjnych zbadany został wpływ struktury krajobrazu opisanej m. in. gradientem udziału upraw rzepaku wokół gniazd pszczół na jakość i skażenie bazy pokarmowej pszczół, parametry populacyjne dwóch pokoleń pszczół oraz ich wrażliwość na przedstawicieli głównych grup insektycydów (tj. pyretroidu Sherpa 100 EC i fosforoorganicznego Dursbanu 480 EC) (artykuły I i II). Zbadany został także wpływ interakcji pomiędzy insektycydami na przeżywalność dorosłych samic *O. bicornis* oraz na wybrane parametry biochemiczne (aktywność trzech enzymów i poziom ATP) (artykuły III i IV).

Hipotezy, które w związku z wyżej wymienionymi celami zostały przetestowane, to:

H1: Wzrastający udział rzepaku wokół gniazda pszczół oraz spadek heterogeniczności krajobrazu wpływa negatywnie na parametry populacyjne pszczół (artykuł I).

H2: Wrażliwość pszczół *O. bicornis* na insektycydy jest wyższa na stanowiskach bardziej zdominowanych przez rzepak (artykuł I).

H3: Wraz ze wzrostem udziału rzepaku wokół gniazd pszczół *O. bicornis* wzrasta stężenie pestycydów w pyłku gromadzonym przez pszczoły jako pokarm dla rozwijających się w gnieździe larw oraz maleje różnorodność pyłku (artykuł II).

H4: Mieszaniny kilku pestycydów wpływają na przeżywalność i parametry biochemiczne *O. bicornis* inaczej niż wynikałoby to z sumarycznego działania tych pestycydów aplikowanych pojedynczo (artykuł III i IV).

METODY

1. Wybór i charakterystyka obszarów do badań (artykuł I i II):

Badania terenowe zostały przeprowadzone w województwach opolskim (2019) i dolnośląskim (2020) i skupiły się na analizie wpływu struktury krajobrazu rolniczego z gradientem udziału rzepaku w najbliższym sąsiedztwie gniazd na przeżywalność dwóch pokoleń pszczół samotnic *Osmia bicornis* i ich wrażliwość na dodatkowy czynnik stresowy – insektycyd. W 2019 roku, 12 gniazd z kokonami rozmieszczono na obrzeżach pól rzepaku o różnej wielkości, reprezentujących zakres pokrycia rzepakiem (ang. *oliseed rape coverage*, ORC) od 6% do 65% w obszarze o promieniu 500 m od gniazda. Dodatkowym kryterium wyboru stanowisk (obszarów o różnym udziale upraw rzepaku w promieniu 500 m wokół gniazda), była ich lokalizacja w krajobrazie zdominowanym przez rolnictwo, czyli położenie w centrum obszaru o wymiarach 5×5 km, w którym pokrycie gruntami rolnymi było powyżej 50% (57-94%), przy czym ponad połowa (>55%) tych gruntów była zdominowana przez duże (>5 ha) pola. Na podstawie danych z BDOT10K oraz informacji o typach upraw otrzymanych z ARiMR i uzupełnionych zdjęciami satelitarnymi, lokalna struktura krajobrazu wokół każdego gniazda (tj. w buforze o promieniu 500 m oraz 1000 m od gniazda) została opisana przede wszystkim wspomnianym procentowym udziałem rzepaku, ale także przez 12 cech krajobrazu (m.in. udział roślinności przy ciekach i zbiornikach wodnych, udział roślinności przy zabudowie, udział krzewów, lasów, ogrodów, pól uprawnych, udział innych niż rzepak upraw kwitnących). Ponadto policzono dwa wskaźniki liniowe: ff – ang. *field-to-field*, czyli długość granic między polami jako przybliżenie średniej wielkości działki i fragmentacji terenu, oraz fn – ang. *field-to-natural*, czyli długość granic między polami a naturalnym elementem krajobrazu. Tych 14 elementów (z wyłączeniem udziału rzepaku) poddano analizie czynnikowej, która wykazała istnienie dwóch głównych gradientów zmiennych krajobrazowych wśród badanych stanowisk. W buforze 500 m pierwszy czynnik (FA1) wyjaśnił 32,4% całkowitej zmienności cech krajobrazu i scharakteryzował krajobraz według cech związanych z terenami zabudowanymi (tj. beton, budynki, ale także roślinność w pobliżu infrastruktury i sady) w konfrontacji z bardziej półnaturalnymi cechami krajobrazu, takimi jak roślinność w pobliżu wody, natomiast drugi czynnik (FA2) wyjaśnił 21,0% całkowitej wariancji i uchwycił przewagę cech „gruntów ornych" (tj. zboża oraz rośliny niekwitnące i kwitnące, ale także krzewy) w konfrontacji z elementami naturalnymi krajobrazu (zbiorniki wodne i roślinność w pobliżu, łąki, lasy oraz długość granic między polami a siedliskami przyrodniczymi). Dodatkowo każde stanowisko

zostało opisane przez wskaźnik różnorodności krajobrazu (ang. *Landscape Diversity Index*, LDI, obliczany jako exp(H'), gdzie H' jest indeksem różnorodności Shannona-Wienera).

2. Badania terenowe prowadzone w latach 2019-2020 (artykuł I)

Zakupione kokony pszczół (pokolenie rodzicielskie P) wraz z formatkami do zasiedlenia i budowy gniazd zostały rozmieszczone w terenie na początku kwitnienia rzepaku (17 kwietnia) i zebrane pod koniec kwitnienia rzepaku (4 czerwca). Każde gniazdo składało się z 16 elementów (formatek), które ułożone jeden na drugim tworzyły wspólnie 360 „rurek" gniazdowych możliwych to zasiedlenia przez pszczoły. Po zebraniu gniazd z terenu, w gniazdach policzono wszystkie komórki założone przez pszczoły, a następnie połowa każdego gniazda (8 formatek) wraz z pyłkiem znajdującym się w komorach gniazdowych została zamrożona, aby zebrać dane na temat jakości i zanieczyszczenia pokarmu zebranego przez samice z pokolenia P dla swojego potomstwa (artykuł II). Druga część gniazd wraz z larwami była przechowywania w komorach klimatycznych, w zmiennych warunkach temperaturowych symulujących temperaturę w terenie, w celu hodowli pszczół (pokolenie F1). W ciągu 3 miesięcy zimowych (styczeń – marzec 2020 r.) kokony zostały wypreparowane z formatek, a następnie zważone i umieszczone pojedynczo w probówkach typu Eppendorf. Na podstawie masy kokonów, wybrane zostały te, które przeznaczono do dalszych badań terenowych. Wybrano po 100 kokonów na gniazdo – 50 kokonów o największej masie i jednocześnie tych z komórek położonych najgłębiej w rurkach i 50 kokonów o najmniejszych masach, pochodzących z komórek w zewnętrznej części rurki, tak aby zwiększyć prawdopodobieństwo otrzymania równej proporcji płci samic (kokony o dużej masie) i samców (kokony o małej masie). Wybrane kokony wraz z gniazdami zostały przewiezione w kwietniu 2020 r. na stanowiska kontrolne (Nadleśnictwa Zawadzkie i Prószków), czyli tereny naturalne, pozbawione pól uprawnych (obrzeża łąk śródleśnych, których udział w promieniu 500 m wynosił od 2 do 31%), upewniając się, że gniazdo jest zlokalizowane w centrum obszaru 5×5 km z mniejszym niż 10% udziałem pól uprawnych i znaczącym udziałem obszarów naturalnych (86-97%), w tym udziałem lasów powyżej 75%. Do tych badań wybrano kokony z 9 gniazd ze względu na niewystarczającą liczbę kokonów w trzech pozostałych gniazdach. Gniazda ze stanowisk kontrolnych zostały zebrane z terenu w lipcu 2020 r. i przechowywane były w komorze klimatycznej w zmiennych warunkach temperaturowych w celu hodowli kolejnego pokolenia pszczół (pokolenie F2), w sposób analogiczny jak to miało miejsce rok wcześniej.

3. Badania parametrów populacyjnych i wrażliwości pszczół *O. bicornis* na pestycydy (artykuł I):

W kwietniu 2020, kokony pochodzące z 12 gniazd, po przezimowaniu zostały przeniesione do temperatury 20°C i pozostawione na ponad dwa tygodnie w celu wygryzienia się dorosłych pszczół z kokonów. Sprawdzono liczbę osobników wygryzionych, czas jaki zajęło im wygryzienie z kokonów oraz płeć, a następnie przeznaczono je do testów ekotoksykologicznych – przeprowadzono dwa testy na samcach i dwa testy na samicach. Układ eksperymentalny obejmował po 60 pszczół danej płci (30 osobników eksponowanych na pestycyd oraz 30 w kontroli) na gniazdo, chyba że mniejsza liczba pszczół w niektórych gniazdach to uniemożliwiała. Pestycyd aplikowany był topikalnie poprzez aplikacje 1µl roztworu pestycydu w 0.01% Tritonie (lub tylko 0.01% Tritonu w przypadku kontroli) na przedplecze pszczoły przy użyciu strzykawki Hamiltona. W testach ekotoksykologicznych sprawdzano przeżywalność pszczół po narażeniu na insektycyd Dursban 480 EC (samce – 0,25 × RAC; samice – 0,2 × RAC, gdzie RAC to zalecane stężenie w dawce polowej, ang. *Recommended Application Concentration*) lub insektycyd Sherpa 100 EC (samce i samice - 1 × RAC). W kolejnym roku (2021) przeprowadzono podobne analizy na pokoleniu F2, z tym że w przypadku testów ekotoksykologicznych możliwe było przeprowadzenie tylko jednego testu (testowano Dursban 480 EC) na samicach pochodzących z 5 gniazd (po 10-30 samic na zabieg (insektycyd lub kontrola) na gniazdo), ze względu na ogólnie znacznie mniejszą liczbę pszczół dostępnych w tym pokoleniu. Ponadto, wszystkim samcom z pokolenia F2 aplikowano tylko 0,01% Triton X-100 (kontrola).

Następujące cechy życiowe zostały zmierzone i uwzględnione w analizie statystycznej dla pszczół pokoleń F1 i F2 dla każdego gniazda: liczba komórek, liczba kokonów, średnia masa kokonów [mg], wskaźnik wygryzania się osobników dorosłych z kokonów [%], średni czas do wygryzienia się (średnia liczba dni potrzebna do wygryzienia się z kokonów, osobno dla samic i samców, po przeniesieniu kokonów do temperatury 4°C, [dni]) oraz stosunek płci osobników dorosłych (samice:samce, F:M). Wrażliwość pszczół na Dursban 480 EC została wyrażona dla każdego gniazda jako mediana czasu życia (LT_{50}), obliczona przy użyciu analizy przeżywania Kaplana-Meiera. Statystyczna istotność zależności między każdą z badanych cech historii życia, w tym wrażliwością na insektycyd (LT_{50}) pszczół z każdego pokolenia (F1 lub F2), a zmiennymi krajobrazowymi (ORC, wartości osi FA1 i FA2, LDI) została przetestowana za pomocą analizy regresji wielokrotnej oddzielnie dla buforów 500 m i 1000 m. Dodatkowo, udział łąk i lasów [%] opisujący lokalny krajobraz wokół gniazd (osobno dla 500 m i 1000 m)

w krajobrazie nierolniczym został użyty jako zmienna objaśniająca dla cech historii życia *O. bicornis* z pokolenia F2. W ten sposób przetestowano nie tylko wpływ krajobrazu pochodzenia rodziców (tj. samic budujących gniazda wzdłuż gradientu pokrycia rzepakiem) na cechy historii życia kolejnych pokoleń (F1 i F2), ale także ewentualny bezpośredni wpływ krajobrazu naturalnego na pszczoły z pokolenia F2.

4. **Analiza pyłku (artykuł II):**

Pyłek pochodzący z połowy każdego gniazda został zebrany z każdej komórki gniazdowej i był przechowywany w -20°C. Następnie, został on wymieszany w obrębie gniazda i podzielony na próbki przeznaczone do analizy pozostałości pestycydów w pyłku (~30 g z każdego gniazda), analiz palinologicznych (~3 g) i kalorymetrycznych (~0.4 g). Analizy poziomów pestycydów w pyłku przeprowadzone zostały przy użyciu technik LC-MS/MS lub GC-MS/MS przez Instytut Ochrony Roślin, Państwowy Instytut Badawczy w Laboratorium Bezpieczeństwa Żywności i Pasz w Białymstoku. Analizy palinologiczne zostały wykonane przez specjalistę z Uniwersytetu Lubelskiego. Wartość energetyczną uprzednio wysuszonych próżniowo próbek pyłku zmierzono za pomocą kalorymetru Semimicro 6725 z termometrem kalorymetrycznym 6772 i bombą tlenową Semimicro 1109A (Parr Instrument Company).

Dla każdej zmiennej zależnej, a mianowicie różnorodności kwiatowej pyłku (ang. *Pollen effective number of species*, PENS), indeksu toksyczności (ang. *Toxic Unit*, TU) i wartości energetycznej pyłku, analizowano zależności ze zmiennymi krajobrazowymi ORC, FA1, FA2 i LDI przy użyciu analizy regresji wielokrotnej, oddzielnie dla buforów 500 m i 1000 m. Ponadto, zależność między PENS a TU została przeanalizowana przy użyciu regresji zredukowanej osi głównej (ang. *reduced major axis*, RMA) w celu sprawdzenia, czy zmniejszona różnorodność pyłku powoduje wzrost jego toksyczności. Analiza RMA została również wykorzystana do przetestowania związku między PENS a wartością energetyczną pyłku. Ponadto wykonano analizę redundancji (RDA) z testem Monte Carlo z 499 nieograniczonymi permutacjami, aby określić wzorzec powiązań pomiędzy stężeniami pestycydów w pyłku a udziałem taksonów roślinnych w gniazdach.

5. **Badania nad wpływem interakcji pomiędzy pestycydami na przeżywalność dorosłych pszczół *O. bicornis* (artykuł III):**

Do badań nad wpływem mieszaniny pestycydów zostały przeznaczone samice pszczół pochodzące z zakupionych kokonów. Wykonano trzy eksperymenty, w których badano wpływ dwuskładnikowych mieszanin insektycydów (Dursban 480 EC × Sherpa100 EC, Sherpa 100

EC × Mospilan20 SP, Karate Zeon 050 CS × Closer) na przeżywalność dorosłych samic. Każdy eksperyment prowadzono w pełnym układzie eksperymentalnym, z pięcioma stężeniami każdego z badanych insektycydów w 0.01% Tritonie X-100 i dodatkowym zabiegiem kontrolnym (pszczoły nie eksponowane na (0,01% Triton X-100). Po indywidualnej topikalnej aplikacji pestycydu, pszczoły pochodzące z tego samego zabiegu (po 20-30 osobników na zabieg w zależności od eksperymentu) przeniesiono do plastikowych terrariów (30×19,5×20,5 cm) i umieszczono w komorze klimatycznej (20±2°C, 60±5% wilgotność względna, fotoperiod 16:8 godzin światło:ciemność) w celu obserwacji przeżywalności. Pszczoły były sprawdzane codziennie i karmione *ad libitum* 33% (w/w) roztworem sacharozy umieszczonym w próbówkach typu Eppendorf.

Ze względu na brak spełnienia założenia o rozkładzie normalnym, do przetestowania wpływu pestycydów i ich interakcji na przeżywalność (czas życia, dni) *O. bicornis* wykorzystano dwukierunkową analizę PERMANOVA z 9999 permutacjami. Ponadto krzywe przeżywania wyznaczone dla każdego zabiegu przy użyciu analizy p Kaplana-Meiera porównywano pomiędzy zabiegami za pomocą testu Log-rank. Wyznaczono także mediany czasu życia (LT_{50}) dla każdego zabiegu.

6. Badania nad wpływem insektycydów na biomarkery narażenia u pszczół *O. bicornis* (artykuł IV):

Do przeprowadzenia eksperymentu zostały użyte pszczoły (samice) pochodzące z zakupionych kokonów. Przeprowadzono dwa identyczne eksperymenty, w krótkim odstępie czasu (9 dni) z narażeniem topikalnym pszczół na trzy insektycydy – Dursban 480 EC, Sherpa 100 EC, Mospilan 20 SP, z trzema stężeniami każdego insektycydu, co w pełnym układzie eksperymentalnym (ang. *full-facorial design*) dało 27 zabiegów, po pięć terrariów na zabieg z co najmniej pięcioma pszczołami w każdym. Osobniki do analiz enzymów (pomiary aktywności AChE (acetylocholinoesteraza), GST (transferaza glutationowa) i EST (esteraza), pierwszy eksperyment) oraz poziomu ATP (drugi eksperyment), pobierane były po 1, 2, 4 i 7 dniu od narażenia i po zamrożeniu w ciekłym azocie przechowywane były w temperaturze -70°C. Wybrane biomarkery odgrywają kluczowe funkcje w metabolizmie, usprawniając proces detoksykacji środków owadobójczych i są mediatorami reakcji na stres oksydacyjny. Wpływ insektycydów, dnia poboru pszczół do analizy i interakcji między tymi zmiennymi na badane biomarkery analizowano za pomocą ogólnych modeli liniowych (ang. *General Linear Models*, GLMs).

WYNIKI

W pierwszej pracy (artykuł **I**) stwierdzono, że udział rzepaku wokół gniazda (ORC) miał wpływ na pszczoły z pokolenia F1, zmniejszając ich sukces wygryzania się z kokonów i czyniąc je bardziej wrażliwymi na Dursban 480 EC. Istotny był też wpływ ORC na średni czas wygryzania się samic F1 z kokonów – im więcej rzepaku, tym samice wygryzały się szybciej. Ponadto, czas wyjścia samic z kokonów skracał się na stanowiskach bardziej zdominowanych przez tereny uprawne (uprawy zbożowe, inne kwitnące bądź niekwitnące uprawy). Wspomniane efekty zniknęły w pokoleniu F2, czyli w pokoleniu, które rozwijało się już na obszarach bez presji rolniczej, u którego istotnym efektem był wzrost udziału samic wraz ze wzrostem ORC. Stwierdzono również, że wraz ze wzrostem udziału rzepaku na stanowiskach gniazdowania pokolenia P mediana czasu życia (LT_{50}) nowo wygryzionych samców F1 zmniejszała się, ale odwrotną zależność stwierdzono dla samców F2. Wyniki wskazują, że rozwój larwalny w warunkach monokultury rzepaku miał negatywny wpływ na niektóre cechy historii życiowej w późniejszym stadium dorosłym („efekt przeniesienia"), ale efekty te w zasadzie zanikły w kolejnym pokoleniu, rozwijającym się w krajobrazie pozbawionym presji z estrony rolnictwa.

W drugiej pracy (artykuł **II**) wykazano, że pszczoły zebrały pyłek z 28 taksonów roślin (6-15 na gniazdo), a w ich zapasach pyłkowych dominowały *Brassica napus* (6,0-54,2%), *Quercus sp*. (1,2-19,4%), *Ranunculus sp*. (0,4-42,7%), Poaceae (1,2-59,9) i *Acer sp*. (0,6-42). W pyłku wykryto pozostałości 12 pestycydów, przy czym acetamipryd, azoksystrobina, boskalid i dimetoat były najczęściej wykrywane. Różnorodność florystyczna i wartość energetyczna pyłku, zależały od struktury krajobrazu. W badanym krajobrazie ryzyko związane z obecnością pozostałości pestycydów w pyłku zmniejszało się wraz ze wzrostem jego różnorodności florystycznej.

W trzeciej pracy (**III**), wbrew zakładanym oczekiwaniom, wykazano, że badane insektycydy oddziałują na przeżywalność samic *O. bicornis* antagonistycznie, a nie synergistycznie. Interakcje antagonistyczne wystąpiły w mieszaninach Sherpa 100 EC × Mospilan 20 SP (zarówno w stężeniach niższych i wyższych niż zalecana dawka polowa) i Karate Zeon 050 CS × Closer (szczególnie, gdy jeden lub oba insektycydy były stosowane w stężeniach odpowiadającym dawkom polowym). Nie stwierdzono interakcji pomiędzy insektycydami Dursban 480 EC × Sherpa 100 EC, najpewniej ze względu na ogólnie wysoką toksyczność Dursbanu 480 EC dla pszczół, nawet w stężeniach znacznie poniżej tych odpowiadających dawkom polowym. Obie mieszaniny, w których stwierdzono interakcje antagonistyczne,

zawierały insektycyd z grupy pyretroidów, których mechanizm działania polega na zakłócaniu bramkowanych napięciem kanałów sodowych, a w konsekwencji zaburzaniu przekazywania sygnałów elektrycznych w układzie nerwowym, powodując subletalny paraliż z efektem „powalającym" (ang. „*knockdown" effect*) i niezdolność do lotu (Krief, 2021), w połączeniu z neonikotynoidem lub sulfoksaminem, których sposób działania polega na zakłócaniu inicjacji sygnałów elektrycznych w neuronach postsynaptycznych, powodując nadmierną stymulację aktywności neuronów, co może być śmiertelne (Seifert, 2014).

W czwartej pracy (**IV**) wykazano wpływ insektycydów Dursban 480 EC i Sherpa 100 EC na inhibicję aktywność AChE i EST. Ponadto, narażenie pszczół na insektycyd Sherpa 100 EC doprowadziło do znacznego wzrostu aktywności GST. Insektycyd Dursban 480 EC zmniejszył pozytywny wpływ Sherpa 100 EC na poziomy ATP, a jego wpływ uwidaczniał się wraz z upływem czasu od narażenia. Dursban 480 EC zmniejszył również pozytywny wpływ Mospilanu 20 SP na poziom ATP. Ponadto obecność Dursbanu 480 EC spowodowała zanik antagonistycznego efektu pomiędzy insektycydami Mospilan 20 SP i Sherpa 100 EC.

WNIOSKI I PODSUMOWANIE

Przeprowadzone badania (artykuł **I**) pokazały, że naturalne elementy krajobrazu, takie jak zbiorniki wodne, roślinność, łąki, lasy oraz długość granic pomiędzy polami a naturalnymi elementami krajobrazu, wpływają pozytywnie na liczbę komórek założonych przez pszczoły *O. bicornis*, zwiększają przeżywalność samców z pokolenia F1 oraz skracają czas wygryzania się samic z pokolenia F1. Ten ostatni parametr jest jednak trudny do zinterpretowania: z jednej strony szybkie wygryzienie z kokonów można rozpatrywać jako zjawisko pozytywne – skraca się czas przebywania pszczół w nieaktywnym stadium, w którym dochodzić może do ubytku masy ciała, więc im szybciej pszczoły się wygryzą, tym mają większą masę ciała i przez to możliwość dłuższego życia (Slominski i Burkle, 2019); z drugiej strony, nie można wykluczyć, że szybkie wygryzanie z kokonów może odbywać się pewnym kosztem metabolicznym w postaci utraty masy ciała, wyczerpania ciała tłuszczowego i przez to krótszą żywotność (Bosch i Kemp, 2000). Znaczenie elementów naturalnych i półnaturalnych dla pszczół samotnych jest zgodne z wcześniejszymi badaniami, które wykazały, że utrata siedlisk półnaturalnych jest jednym z głównych czynników powodujących spadek liczebności zapylaczy (Ricketts i in., 2008; Tscharntke i in., 2012). Rozwój larw w warunkach monokultury rzepaku wpływał niekorzystnie na niektóre cechy w późniejszym stadium dorosłym, ale w zasadzie zanikał w kolejnym pokoleniu, gdyż efekt matczyny przejawiał się tylko w zmianie proporcji płci potomstwa na korzyść samic. Efekt ten można rozpatrywać jako korzystny z punktu widzenia ochrony zapylaczy w krajobrazie rolniczym – „produkcja" samic jest bardziej kosztowną inwestycją z punktu widzenia samicy matki, a zatem można wnioskować, że już w pierwszym pokoleniu po zmianie środowiska życia na bardziej korzystne (tj. zmiana krajobrazu rolniczego zdominowanego przez uprawy rzepaku na tereny nierolnicze z obecnością łąk), pszczoły są w stanie odbudować swoje populacje. Uzyskane wyniki wskazują na duże znaczenie warunków rozwoju pszczół w krajobrazie rolniczym w ochronie pszczół i ocenie ryzyka – obecność takich elementów krajobrazu jak zbiorniki wodne wraz z otaczającą je roślinnością, łąki, lasy oraz struktura krajobrazu charakteryzująca się dużą długością granic między polami a siedliskami naturalnymi, powinny być uwzględnione w ochronie owadów pożytecznych w krajobrazach rolniczych.

Przeprowadzone analizy pyłku (artykuł **II**) wykazały, że samice *O. bicornis*, nawet w krajobrazie z dużym udziałem upraw rzepaku poszukują pyłku innych gatunków roślin dla swojego potomstwa, w tym pyłków drzew i krzewów. Zarówno różnorodność florystyczna jak

i wartość energetyczna komórek pyłkowych zależały od struktury krajobrazu w najbliższym sąsiedztwie gniazda (bufor 500 m), co jest zgodne z dystansem, jaki zwykle pokonują pszczoły w poszukiwaniu pokarmu (Gathmann and Tscharntke, 2002). Ponadto nawet strukturalnie prosty krajobraz może zapewnić różnorodne pożywienie dla *O. bicornis*, jeśli gniazdo znajduje się w pobliżu pojedynczego, ale zróżnicowanego pod względem zasobów płatu roślinności. Jednak zarówno uprawy masowo kwitnące jak i pobliskie kwiaty, krzewy i drzewa mogą być skażone szeroką gamą pestycydów – stężenia pestycydów w komórkach pyłkowych były skorelowane zarówno z zawartością pyłku roślin uprawnych (*Bnapus*), jak i nieuprawnych (np. *Ranunculs sp*., Poaceae, *Carex sp*.). W badanym krajobrazie ryzyko związane z obecnością pozostałości pestycydów w pyłku (wyrażone wskaźnikiem toksyczności „Toxuc Unit") ogólnie malało wraz ze wzrostem różnorodności florystycznej pyłku. Zapewnienie pszczołom zróżnicowanej bazy pokarmowej w krajobrazie rolniczym powinno być uwzględnione w strategiach dotyczących ochrony zapylaczy. Z kolei ocena ryzyka, jakie pestycydy stanowią dla zapylaczy, powinna uwzględniać wpływ mieszanin pestycydów. Ten wpływ na przeżywalność, jak pokazują otrzymane wyniki (artykuł **III**), nie musi być synergistyczny, ale może prowadzić do efektów mniejszych niż wynikałoby to z sumy efektów pojedynczych substancji. Warto zauważyć, że antagonistyczne interakcje zachodziły pomiędzy dwoma insektycydami, z których jeden oddziaływał na zaburzanie przekazywania sygnałów elektrycznych w układzie nerwowym (pyretroid), a drugi na zakłócanie inicjacji sygnałów elektrycznych w neuronach postsynaptycznych (neonikotynoid lub związek z grupy sulfoksymin) (tj. Sherpa × Mospilan" i „Karate × Closer"). Podkreśla to złożoność działania mieszanin pestycydów na owady pożyteczne. Wpływ mieszanin insektycydów w stężeniach subletalnych widoczny był w oddziaływaniu na badane enzymy (AChE, GST, EST) i poziom ATP (**IV**) – w przypadku ATP dając wyniki, których nie dałoby się przewidzieć testując każdy z insektycydów osobno. Wykazano, że enzymy AChE i EST są odpowiednimi markerami narażenia nie tylko na pestycydy fosforoorganiczne, ale także na pyretroidy, natomiast GST wydaje się być wiarygodnym markerem w badaniach subletalnego wpływu pyretroidów na pszczoły samotne. Najbardziej czułym biomarkerem był poziom ATP, który wykazywał złożony wzorzec odpowiedzi. Biorąc pod uwagę fakt, że narażenie na Sherpa 100 EC oraz Dursban 480 EC zmniejszało aktywności AChE i EST, a równocześnie narażenie na Sherpa 100 EC prowadziło do wzrostu poziomu ATP, mogło dojść do uaktywnienia niektórych szlaków metabolicznych (widoczne jako wzrost poziomu ATP) i zahamowania innych (widoczne jako spadek aktywności enzymów). Obecność insektycydu Dursban 480 EC w mieszaninie upośledzała produkcję ATP, a biorąc pod uwagę fakt, że insektycyd ten zwiększał

śmiertelność pszczół, nawet w stężeniach znacznie niższych niż zalecane do stosowania w terenie, wyniki sugerują, że pestycydy fosforoorganiczne nie powinny być mieszane z neonikotynoidami i/lub pyretroidami.

Podsumowując, wyniki uzyskane w niniejszej rozprawie, zwiększając naszą wiedzę na temat zagrożeń stwarzanych przez intensyfikację rolnictwa dla pszczół innych niż *Apis*. Otrzymane wyniki, oprócz znaczenia poznawczego, będą również wykorzystane praktycznie do opracowywania i weryfikacji modeli matematycznych wykorzystujących platformę ALMaSS, pozwalających na poprawę stanu środowiska rolniczego poprzez odpowiednie zarządzanie strukturą terenów rolniczych przy zachowaniu wysokiej wydajności rolniczej (Ziółkowska i in., 2021, 2023). Ponadto, stwierdzenie istotnych interakcji pomiędzy insektycydami i ich wpływu na pszczoły samotne potwierdza, że niezbędne jest wprowadzenie zasadniczych zmian w procedurach testowania środków ochrony roślin, które obecnie obligatoryjnie są wykonywane wyłącznie dla pojedynczych substancji chemicznych.

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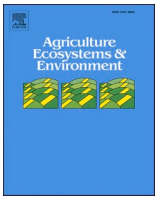
Misiewicz, A., Mikołajczyk, Ł., Bednarska, A.J., 2023. Impact of oilseed rape coverage and other agricultural landscape characteristics on two generations of the red mason bee *Osmia bicornis*. Agriculture, Ecosystems & Environment 352: 108514. https://doi.org/10.1016/j.agee.2023.108514 (IF = 6,6, 200 pkt MNiSW).

Agriculture, Ecosystems and Environment 352 (2023) 108514

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee



Impact of oilseed rape coverage and other agricultural landscape characteristics on two generations of the red mason bee *Osmia bicornis*

Anna Misiewicz [a,*], Łukasz Mikołajczyk [a,b], Agnieszka J. Bednarska [a]

[a] Institute of Nature Conservation, Polish Academy of Sciences, A. Mickiewicza 33, 31-120 Kraków, Poland
[b] Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

ARTICLE INFO

Keywords:
Solitary bee
Agroecosystem
Insecticide
Carry-over effect
Maternal effect

ABSTRACT

The intensification of agriculture and the related increase in pesticide use and land transformation toward large-scale monocultures are linked to a global insect decline that impacts biodiversity and essential ecosystem services. Apart from direct effects, potential delayed carry-over and maternal effects from past exposure to intensive farming at different life stages may have profound implications for population dynamics. We studied the effects of farming of varying intensity, represented by different proportion of oilseed rape in the close vicinity of nests and, at the same time, by different landscape structure on the life history traits of two generations of the red mason bee (*Osmia bicornis*) and the sensitivity of bees toward insecticide. Twelve *O. bicornis* nests with cocooned adults of the parental (P) generation were located at sites representing 6–65% of oilseed rape coverage (ORC, % land cover) within nonoverlapping circles of 500 m radius. The bees were allowed to build their nests during the entire period of oilseed blooming. The following year, part of the newly emerged bees (generation F1) was used to test sensitivity to Dursban 480 EC insecticide, and the remaining bees were transferred to mid-forest meadows and allowed to establish the next (F2) generation in the areas without agricultural pressure. The F2 adults were tested the following year to determine their sensitivity to the same insecticides as F1 adults. We showed that ORC affected the F1 bees by decreasing their emergence success, shortening the emergence time of F1 females, and making them more sensitive toward Dursban 480 EC (topical exposure). However, these effects of ORC and the effect of landscape structure around the nests on emergence time disappeared in the F2 generation that developed in the areas without agricultural pressure. The only significant effect observed in F2 bees was the increase in the female:male ratio with increasing ORC. We also found that with increasing ORC, the survival time of newly emerged F1 males decreased, but the opposite relationship was found for F2 males. The results indicate that larval development under monoculture farming has some carry-over effects, but the effects mostly disappear in the next generation. Implications of carry-over and maternal effects for population sustainability should be considered in pollinator conservation and management decisions to mitigate the effects of agricultural landscape.

1. Introduction

In recent years, a worldwide decline in the number of insects has been observed (Hallmann et al., 2017; IPBES, 2018; Potts et al., 2010; Seibold et al., 2019), with a third of them being threatened with extinction (Sánchez-Bayo and Wyckhuys, 2019). Particularly noteworthy is the decrease in the number of pollinators (e.g., 33% of wild pollinator species have decreased between 1980 and 2013 in Britain) (Powney et al., 2019). Plant pollination by insects is a key ecosystem service necessary for the production of most crops (IPBES, 2016), worth €153 billion and representing 9.5% of the world agricultural production value used for human consumption (data from 2005) (Gallai et al., 2009). Among pollinators, western honey bees (*Apis mellifera* L.) provides the most highly valued pollination services for a wide variety of agricultural crops (Calderone, 2012) and therefore has received the highest amount of attention and research focus. Although the role of *A. mellifera* in the environment is invaluable, Garibaldi et al. (2013) showed that honey bees do not maximize pollination and do not replace the contributions of diverse wild insect assemblages to fruit set for a broad range of crops on all continents with farmland. Approximately 20% of the pollination services in agricultural production are provided by wild bees (Losey and Vaughan, 2006). Wild insects also pollinate

* Corresponding author.
E-mail address: misiewicz@iop.krakow.pl (A. Misiewicz).

https://doi.org/10.1016/j.agee.2023.108514
Received 10 May 2022; Received in revised form 23 March 2023; Accepted 3 April 2023
Available online 10 April 2023

selected crops more effectively than honey bees (Garibaldi et al., 2013). Among wild pollinators, solitary bees of the genus *Osmia* proved to be effective pollinators in several crops across the world, such as apples and cherries (Gruber et al., 2011; Ryder et al., 2020; Sekita, 2001), strawberries (MacInnis and Forrest, 2019; Herrmann et al., 2019), sunflowers (Mallinger et al., 2019) and in Europe, mainly in oilseed rape cultivations (*Brassica napus*) (Holzschuh et al., 2013).

Several factors are responsible for the decline in pollinators, but one of the main causes is the intensification of agriculture and the related transformation of land use and farming practices, such as the use of pesticides (Dudley and Alexander, 2017; Vanbergen and Initiative, 2013). Intensive development of agriculture promotes large-scale agricultural monocultures, resulting in the decline in heterogeneity of the landscape and seminatural habitats (buffer strips and wastelands), leading to the loss of nesting and foraging resources for pollinators and increased exposure to insecticides (Kline and Joshi, 2020; Long and Krupke, 2016). Large monocultures of crops attractive to pollinators, such as oilseed rape (*Brassica napus* L), are potentially hazardous (Holzschuh et al., 2011), also due to use of pesticides (herbicides, insecticides, fungicides), which may have a negative effect on pollinators (Sgolastra et al., 2018). Indeed, residues of many pesticides have been found in the pollen and nectar of flowering crops (Dively and Kamel, 2012), wild flowers growing in agricultural field margins (Botías et al., 2015; David et al., 2016) or food provisions collected by wild bees for their offspring (Woodcock et al., 2017; Bednarska et al., 2022). Wild pollinators often need diverse floral resources for better reproductive output (Klaus et al., 2021). For example, a high proportion of agricultural habitats around *Osmia cornifrons* nests decreased the number of female offspring by reducing pollen diversity in the diet (Centrella et al., 2020). On the other hand, Yourstone et al. (2021) and Holzschuh et al. (2013) showed that *Osmia bicornis* benefits from the proximity of oilseed rape, which is an abundant source of nectar for adults; the reproductive output of bees was higher near oilseed rape and additionally increased with the availability of trees and grasslands in the surroundings, but oilseed rape pollen may be of poor quality for larval development (Dobson et al., 2012).

Due to their invaluable role in the environment and their recognized decline, wild pollinators, including solitary bees, have recently been receiving more attention in pesticide risk assessments (Heard et al., 2017; Peters et al., 2016; Ruddle et al., 2018; Sandrock et al., 2014; Woodcock et al., 2017). In contrast to *Apis mellifera*, which is the standard species both in ecotoxicological tests (Douglas et al., 2020; Thompson and Pamminger, 2019) and in studies on landscape structure effects (Rosas-Ramos et al., 2017), knowledge about the effects of chronic exposure to pesticides on the life history parameters of solitary bees is very limited. Because of their biological and morphological specificity and lack of social lifestyle, solitary bees may be affected by pesticides and/or surrounding landscape characteristics differently than social *Apis* bees (Brittain and Potts, 2011; Uhl and Brühl, 2019). Moreover, organisms chronically exposed to low concentrations of pesticides (or other stressors) may not show signs of acute toxicity (e.g., increased mortality) but may have affected other life history parameters and/or reduced tolerance to other stress factors, such as other chemicals and/or various types of natural environmental factors (e.g., extreme temperatures, shortage of food) (Stone et al., 2001). Thus, stress during one life stage (e.g., larvae) may carry over to affect later life stages (e.g., adults) (Anderson and Harmon-Threatt, 2019; Stuligross and Williams, 2021). In ecological context, carry-over effect is defined as an effect which occurs in any situation in which an individual's previous history and experience explains their current performance in a given situation (O'Connor et al., 2014). Such effects can occur between life-history stages, developmental stages, physiological stages, or social stages, and each is associated with a discrete time-scale (O'Connor et al., 2014). Moreover, the maternal environment may also affect offspring quality (Mousseau and Fox, 1998) and have a delayed impact on the performance of subsequent generations (Mousseau and Dingle, 1991). Understanding bee responses to insecticide pressure in their local landscape context may help in assuring vital habitat conditions to prevent beneficial species from extinction and in management and conservation decisions for pollinators.

The objective of this study was to investigate the interplay of landscape structure and proportion of oilseed rape in the close vicinity of *Osmia bicornis* nests on the life history parameters of the bees and sensitivity of newly emerged adults (F1 generation) toward Dursban 480 EC. Additionally, cocooned adults from the F1 generation were transferred in the following year to natural mid-forest meadows and allowed to establish their populations in the areas without agricultural pressure. In that way, we were able to test for possible carry-over effects from past "agricultural pressure" measured as oilseed rape coverage at the larval stage (i.e., how larval environment affects adult performance) and for maternal effects that may lead to phenotypes of offspring that change their fitness (i.e., how parental environment of F1 generation affects quality of F2 offspring). We selected oilseed rape (winter variety), as it is an important mass-flowering crop in the European Union (5.2 million hectares of oilseed rape was planted in the EU in 2020; Eurostat, 2017) that is attractive to wild bees but treated with a number of pesticides (Goulson et al., 2015). We hypothesized, although the parental generation of bees could potentially benefit from increasing oilseed rape coverage around the nest as a source of nectar, the effect of increasing ORC on the next generation of bees might be negative due to the reduced quality of pollen collected for larvae and/or increased insecticide exposure in that pollen. Such a delayed carry-over effect from past exposure at the larval stage could have a persistent effect on the subsequent generation (F2).

2. Materials and methods

2.1. Selection and characteristics of study areas and study sites

Field studies were conducted in the Opolskie and Lower Silesia districts (Poland) in 2019 and 2020. The region for locating *Osmia bicornis* nests in 2019 was chosen to represent the intensive agricultural (A) landscape. One *O. bicornis* nest with 550 cocooned adults of the parental (P) generation were placed at the perimeter of each of the 12 oilseed rape fields of different sizes, representing the highest possible range of oilseed rape coverage (ORC, 6–65%) within nonoverlapping circles of 500 m radius. Additionally, each study site fulfilled the prerequisite of being located in the center of the landscape of 5 × 5 km with 57–94% agricultural land coverage and with more than half (>55%) of the arable land dominated by large fields (>5 ha) (Table S1, Fig. 1). In turn, the field study on the F1 generation in 2020 was performed in a nonagricultural landscape (N) with less than 10% of the 5 × 5 km landscape around the nest covered by agricultural fields, a significant proportion of natural areas (86–97%) and forest coverage > 75%. Nine out of 12 nests, in which enough *O. bicornis* cocooned adults of the F1 generation were found, were located in the mid-forest meadows, which covered 2–31% of the area within a 500 m radius around the nest (Fig. 1). Meadows located in the mixed Scots pine forest with significant admixtures of oak, beech, spruce, maple and black alder were selected, as it was important that the subsequent (F2) generation of bees will develop far from agricultural areas (i.e., without the pressure of intense agriculture) and with good access to food. The polylectic *O. bicornis* opportunistically collects pollen of plants present in the environment, including pollen from oak, maple, chestnut, and elm trees (Bednarska et al., 2022; Splitt et al., 2021a).

The original identification numbers (1−12) were kept for the nests in both landscapes, with the landscape type encoded as A (agricultural) and N (nonagricultural). For every nest (12 nests in agricultural (A) and 9 nests in nonagricultural (N) landscape) two landscape maps were created for the circular areas of 500 and 1000 m radius (later referred to as "buffers") to match the red mason bee foraging range (Bednarska et al., 2022; Gathmann and Tscharntke, 2002): 1000 m buffer reflected

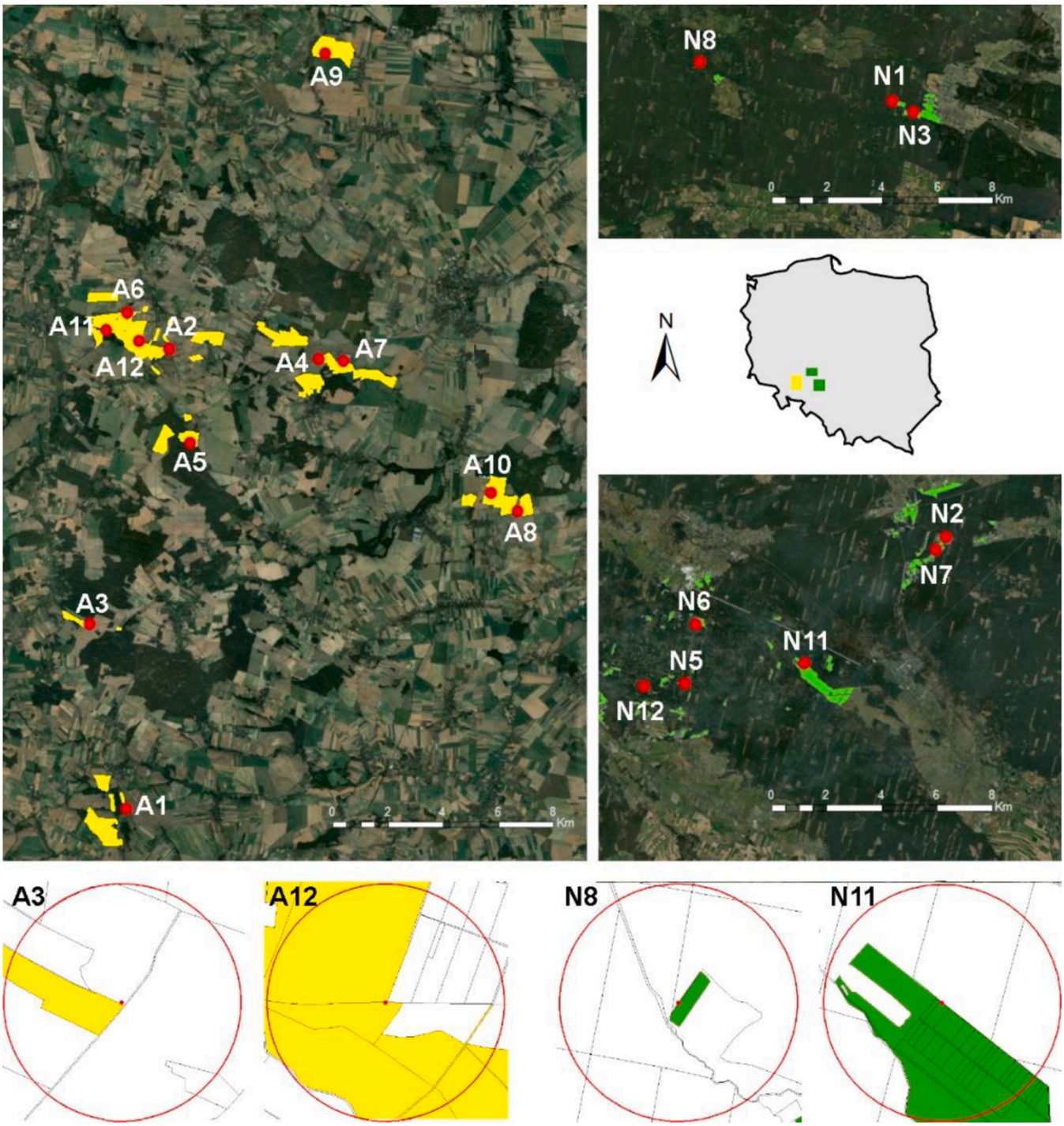


Fig. 1. Location of the 12 study sites in the agricultural (A) landscape and the 9 study sites in the nonagricultural (NA) landscape (red circles) in the Opolskie Voivodeship (Poland) and the examples of the 500 m buffer with the oilseed rape (yellow) or mid-forest meadow (green) coverage indicated for A or NA landscapes, respectively.

the maximum homing distance (the distance at which 10% of *O. bicornis* bees is able to return to the nest (Gathmann and Tscharntke, 2002)) and the area in the close vicinity of the nest (i.e., within the radius of 500 m) is reported to represent the landscape immediately available for *O. bicornis* to roam (Bednarska et al., 2022). As such, both buffers provide the most meaningful and robust explanatory base for *Osmia bicornis* life traits (Bednarska et al., 2022; Mikołajczyk et al., 2021). The maps combined information from two main data sources:

(i) Land cover information was derived from the Polish Vector Database (1:10000) BDOT10k with additional units of vegetation around water bodies and along infrastructure also calculated on the basis of BDOT10k.

(ii) A map of agricultural reference parcels (i.e., cadastral parcels) mapped within the national Land Parcel Identification System LPIS together with information on crop types for 2019 provided by the Polish Agency for Restructuring and Modernization of Agriculture (ARMA), and the missing crop data (ca. 15%) were obtained from satellite imagery (Copernicus Land Monitoring Service accessed through ArcMap via WMS service) by visual examination and comparison to neighboring units.

The vector information layers from (i) and (ii) were converted into raster layers of 1 m spatial resolution, grouped thematically and stacked on each other according to predefined rules, generating a detailed raster land cover map with complete coverage. Land-cover units were subsequently reclassified to create classes of landscape elements that are expected to be functionally equivalent for wild bees (Mikołajczyk et al., 2021). Clustering allowed for a reduction of the unnecessary complexity of the initial land cover maps by distinguishing the following 9 land cover units (1−9): vegetation by water bodies (vegwat), water bodies (wat), concrete and infrastructure (con), vegetation by infrastructure (veginf), bushes (bush), forests (for), buildings (bui), meadows (mead), orchards (orch) and 4 agricultural field units (10−13) grouped based on crop types: cereals (cultivated, anemophilous grasses; cer), nonflowering crops (cultivated plants harvested before blooming or not blooming at all; noflo), flowering crops (cultivated plants harvested after blooming; flo), and oilseed rape (selected separately due to its importance for pollinators; osr; Stanley and Stout, 2013). All 13 discrete, nonoverlapping units, covering the full extent of the area of interest, were represented as a 1 m resolution raster. Additionally, two linear features were calculated: field-to-field borders per total agricultural land (as a proxy for plot size and land fragmentation; ff) and field-to-natural borders (course of borders between agricultural fields and natural habitats, e.g., forest, bushes, etc.; fn). The detailed description of landscape units and raw data are summarized in Table S1 (Supplementary Materials), and for more details, see Mikołajczyk et al. (2021). Spatial data were manipulated with the use of ArcMap 10 (ESRI, 2020).

2.2. Study design

In spring 2019 (17th April), at the beginning of oilseed rape flowering, twelve artificial nests, each with 50 g of *Osmia bicornis* cocoons (equivalent to ca. 550 cocoons) purchased from a local supplier (Pszczelinka, Kapka Sp. z o. o., Poland) were set up on the perimeters of oilseed rape fields. Each nest consisted of 16 nesting cases made of polypropylene, stacked on top of each other, each with 22 or 23 nesting tubes (thus forming 360 nesting tubes of internal size of 7.5 ×7.5 mm) opened at one end only, and were 14.5 cm in length (Pszczelinka, Kapka Sp. z o. o., Poland). Nesting cases (Fig. S1) were placed in box-shaped housing units made of durable and weather-resistant polyethylene (Pszczelinka, Kapka Sp. z o. o., Poland). To protect the bees against birds and rodents, each nest was closed with a plastic mesh and placed on a pole ca. 1 m above the ground (Fig. S1). The entrance to the nest was oriented toward the southeast to enhance *O. bicornis* activity in the early morning (MacIvor, 2017).

During oilseed rape flowering, *O. bicornis* adults emerged from cocoons (parental generation, P) and mated, and then females built brood cells, provisioning them with a mixture of pollen and nectar collected from the surrounding area and laying a single egg on top of each provision (Fig. S1). Immediately after oilseed rape flowering finished (4th June), all nests were brought to the laboratory of the Institute of Environmental Sciences at Jagiellonian University in Kraków, Poland. The total number of sealed cells comprising pollen provision with an egg or larvae was counted, and then half of each nest (8 lower nesting cases) was frozen at − 20 °C for further analysis (not discussed in this paper), while the second half was kept in a walk-in climatic chamber under changing temperature conditions (ca. two months at 20 °C, two months at 15 °C, a month at 10 °C and finally overwintering at 4 °C until April 2020) to breed the F1 generation (Fig. S1). During winter (January - March 2020), the number of produced cocoons was counted. Cocoons were extracted from the nesting cases, weighed to the nearest 0.1 mg and placed individually in labeled Eppendorf tubes with a hole in the lid and stored at 4 °C for further overwintering.

Typically, *O. bicornis* deposits female progeny deeper inside the nest (inner brood cells) and male progeny toward the nest entrance (outer brood cells) (Ivanov, 2006) and female progeny is heavier than male progeny (Radmacher and Strohm, 2010). Based on cocoon weight and position in the nest, 100 cocoons (F1 generation) from each nest were selected for the field study in spring 2020: 50 heavier cocoons originating from the cells located deepest in the nesting tubes, most likely representing females, and 50 lighter cocoons derived from cells in the outer part of the nesting tubes, presumably males. The mean weight of F1 cocoons from nests selected for the field study ranged from 139.7 ± 19.06 to 141.8 ± 8.14 mg for "females" and from 55.6 ± 7.45 to 57.4 ± 3.69 mg (Table 1) for "males".

In spring 2020 (15 April), selected cocoons together with the artificial nests (the same as used in the previous year) were placed in the nonagricultural (N) landscape (i.e., mid-forest meadows). However, due to the insufficient number of cocoons in three nests (Table 1), only 9 out of the original 12 nests could be used in 2020. The nests remained in the field until July 22nd and were then transferred to the laboratory and kept in a climatic chamber under variable temperature conditions as described above to breed the next generation of bees (F2 generation). In January 2021, cocoons were extracted from nesting tubes, weighed and placed individually in labeled Eppendorf tubes with a hole in the lid and stored at 4 °C for further overwintering until April 2021.

The remaining cocoons of the F1 generation (i.e., those that were not taken to the field in 2020) as well as cocoons from the F2 generation (extracted from tubes in 2021) were used to check the emergence success and to test the sensitivity of emerged bees to Dursban 480 EC. In April 2020 (F1 generation) and April 2021 (F2 generation), Eppendorf tubes with cocoons originating from 12 or 9 nests, respectively, were transferred to a temperature 20 °C, 60 ± 5% relative humidity (RH) and 16:8 h light:dark (L:D) regime to emerge. The tubes were controlled daily, and the number of emerged adults, time to emergence [days] and their sex were recorded. Upon emergence, less than 24-h-old bees from each nest were transferred from Eppendorf tubes to respective Plexiglas cages (46 ×30 ×17 cm) with air flow supply from the top, males and females from each nest separately. Adult bees were kept in the cages until enough bees were collected to run the ecotoxicological test (see below). The bees were fed *ad libitum* with sucrose solution 33% (w/w) placed into 2-ml Eppendorf tubes without lids but with cotton wool inside the tube and with a small square-cut yellow sponge cloth mounted around the tube (Fig. S2).

2.3. Sensitivity of bees toward Dursban 480 EC

Dursban 480 EC containing 44.86% chlorpyrifos as an active ingredient (a.i.) was used (Dow AgroSciences, Warszawa, Poland). Although chlorpyrifos was prohibited in the EU in 2020, it is still used in flowering crops outside the EU, potentially threatening pollinating insects (Onwona-Kwakye et al., 2020; Urlacher et al., 2016). Nevertheless, for testing the sensitivity of bees to insecticides, other formulation might as well have been used, especially that we were not interested in the effect of a particular formulation on bees but rather in testing whether the bees from F1 and F2 generation (i.e., the bees that developed under agricultural pressure and their offspring) are handicapped in terms of their resistance to additional stressor (here an insecticide). The recommended field application rate (RAR) given by their manufacturers, together with recommended dilutions (300 L per hectare), were used to calculate the Recommended Application Concentration (RAC) of the agrochemical to prepare experimental solutions with respect to actual concentrations used by farmers in the field. The concentrations of insecticide solution were chosen considering its toxicity to *O. bicornis* females after topical application found by Mokkapati et al. (2021). The following concentrations of the insecticide, prepared in 0.01% Triton X-100 (Sigma–Aldrich, Poland) to facilitate the adhesion of the applied solution to bees, were used: 0.25 RAC and 0.2 RAC for males and females, respectively. The RAC of Dursban 480 EC for females was lowered after a very strong effect was seen for males that were tested before females, as they emerged earlier (Table 1). The 0.2 RAC of Dursban 480 EC (which gives a chlorpyrifos concentration of 0.192 μg/μl) was confirmed by a certified external contractor (Laboratory of Food and Feed Safety at the

Table 1
Population parameters of *Osmia bicornis* from nests located in agricultural landscape (A) in 2019 and nonagricultural landscape (N) in 2020.

Nest ID	No. of cells produced in the whole nest	No. of cells produced in the half of the nest	No. of cocoons	Mean (± SD) cocoon weight [mg]	Mean (± SD) weight of female cocoon [mg] selected for field study	Mean (± SD) weight of male cocoon [mg] selected for field study	Adult emergence rate [%]	Percentage of emerged females [%]	Percentage of emerged males [%]	Sex ratio (F:M)	Average time to emerge for females [day]	Average time to emerge for males [day]
						Agricultural (A) landscape						
A1	837	446	320	93.4 ± 32.16	141.8 ± 8.74	56.3 ± 5.24	91.24	42.57	57.43	0.74	5.6 ± 1.55	1.3 ± 0.54
A2	1071	637	539	94.8 ± 31.05	141.8 ± 4.31	57.4 ± 3.69	89.47	48.10	51.90	0.93	4.3 ± 1.53	1.2 ± 0.52
A3	952	442	284	95.8 ± 32.28	140.9 ± 8.85	57.3 ± 6.94	97.69	40.00	60.00	0.67	5.3 ± 1.30	1.4 ± 0.60
A4	423	77	68	82.7 ± 28.19	-	-	98.46	34.33	65.67	0.52	5.5 ± 1.10	1.1 ± 0.37
A5	554	327	247	91.8 ± 31.30	139.7 ± 19.06	57.3 ± 5.49	96.55	44.37	55.63	0.80	6.2 ± 1.22	1.5 ± 0.82
A6	1074	590	434	88.7 ± 30.45	141.9 ± 8.14	56.5 ± 3.23	89.26	29.80	70.20	0.42	2.4 ± 2.68	1.7 ± 1.74
A7	608	282	263	95.9 ± 31.64	140.7 ± 7.40	55.6 ± 7.45	95.39	31.85	68.15	0.47	5.5 ± 1.57	1.3 ± 0.52
A8	1229	681	484	95.4 ± 32.18	140.4 ± 4.40	56.5 ± 4.28	90.40	38.11	61.89	0.62	4.3 ± 1.76	1.3 ± 0.82
A9	284	56	48	87.0 ± 30.19	-	-	95.45	41.3	58.70	0.70	5.5 ± 1.76	1.4 ± 1.43
A10	473	199	132	88.0 ± 32.42	-	-	81.10	50.00	50.00	1.00	4.5 ± 1.33	1.3 ± 0.74
A11	1749	835	675	91.1 ± 33.10	141.2 ± 4.52	56.9 ± 2.74	82.16	49.04	50.96	0.96	4.1 ± 1.36	1.0 ± 0.76
A12	1607	766	657	94.7 ± 31.13	141.6 ± 3.91	56.9 ± 2.60	89.62	42.00	58.00	0.72	4.4 ± 1.66	1.3 ± 0.59
						Non-agricultural (N) landscape						
N1	97	-	49	93.6 ± 41.81	-	-	63.27	32.26	67.74	0.48	7.5 ± 1.29	3.2 ± 0.68
N2	198	-	178	118.0 ± 30.46	-	-	77.53	65.47	34.53	1.90	7.9 ± 1.70	3.3 ± 1.37
N3	95	-	67	101.4 ± 33.85	-	-	80.60	63.64	36.36	1.75	8.3 ± 1.55	3.5 ± 1.35
N5	82	-	56	78.0 ± 33.20	-	-	28.57	62.50	37.50	1.67	8.3 ± 2.10	3.5 ± 0.76
N6	37	-	17	92.8 ± 28.57	-	-	88.24	80.00	20.00	4.00	8.3 ± 1.42	3.7 ± 0.47
N7	289	-	235	126.6 ± 34.59	-	-	95.32	72.77	27.23	2.67	8.3 ± 1.51	3.7 ± 0.92
N8	232	-	203	106.7 ± 39.36	-	-	96.55	51.01	48.99	1.04	7.4 ± 1.30	3.0 ± 0.73
N12	43	-	30	103.4 ± 23.69	-	-	83.33	80.00	20.00	4.00	7.5 ± 1.32	2.8 ± 0.40

Table 2
Median lethal times (LT_{50}, days) and dry weight (mg) of *Osmia bicornis* females and males from the F1 generation (developed in agricultural (A) landscape) exposed topically to Dursban 480 EC or 0.01% Triton (control). The same lowercase letter indicates no significant differences (at $p \geq 0.004$ after applying the Bonferroni correction for multiple comparisons) in survival (pairwise comparison of survival curves, log-rank test) between exposed and control bees within the nest. Number of bees used is indicated in brackets.

Nest ID	Mean dry weight of females [mg]		$LT_{50} \pm SE$ [days] for females		Mean dry weight of males [mg]		$LT_{50} \pm SE$ [days] for males	
	Control (Triton)	Exposed (Dursban)	Control (Triton)	Exposed (Dursban)	Control (Triton)	Exposed (Dursban)	Control (Triton)	Exposed (Dursban)
A1	34.00 ± 9.054^a (30)	36.89 ± 7.759^a (30)	36 ± 2.15^a (30)	35 ± 2.65^a (30)	20.42 ± 7.812^a (30)	15.46 ± 2.487^a (30)	23 ± 1.08 (30)	$< 1 \pm$ NE (30)
A2	36.29 ± 8.265^a (28)	34.93 ± 10.792^a (30)	38 ± 6.60^a (28)	38 ± 3.64^a (30)	21.02 ± 5.862^a (29)	16.22 ± 2.075^b (30)	21 ± 0.72 (29)	$< 1 \pm$ NE (30)
A3	36.56 ± 7.851^a (30)	34.79 ± 8.343^a (29)	36 ± 1.36^a (30)	42 ± 1.41^a (29)	22.38 ± 7.847^a (30)	16.26 ± 2.195^b (30)	27 ± 0.65 (30)	$< 1 \pm$ NE (30)
A4	39.75 ± 11.562^a (12)	44.54 ± 12.298^a (12)	35 ± 2.89^a (12)	33 ± 1.15^a (12)	19.12 ± 5.259^a (16)	13.39 ± 2.327^a (17)	24 ± 0.77 (16)	$< 1 \pm$ NE (17)
A5	32.52 ± 8.914^a (30)	32.62 ± 9.088^a (30)	40 ± 2.56^a (30)	38 ± 2.95^b (30)	20.72 ± 5.912^a (30)	16.69 ± 3.317^a (30)	26 ± 0.81 (30)	$< 1 \pm$ NE (30)
A6	36.92 ± 9.022^a (28)	33.96 ± 8.270^a (29)	37 ± 2.62^a (28)	33 ± 1.97^a (29)	23.50 ± 6.594^a (30)	16.24 ± 4.065^b (30)	18 ± 0.42 (30)	$< 1 \pm$ NE (30)
A7	37.42 ± 8.227^a (20)	35.46 ± 7.867^a (20)	39 ± 2.98^a (20)	33 ± 5.43^b (20)	24.02 ± 10.107^a (30)	16.55 ± 2.119^b (30)	26 ± 1.12 (30)	$< 1 \pm$ NE (30)
A8	33.49 ± 7.142^a (29)	33.25 ± 7.984^a (30)	35 ± 1.37^a (30)	29 ± 3.42^a (30)	18.87 ± 3.936^a (30)	16.08 ± 2.177^b (30)	22 ± 3.27 (30)	$< 1 \pm$ NE (30)
A9	45.49 ± 10.607^a (10)	34.22 ± 9.914^a (9)	45 ± 1.55^a (10)	33 ± 1.49^a (9)	21.95 ± 8.488^a (8)	13.01 ± 1.610^a (9)	25 ± 0.81 (8)	$< 1 \pm$ NE (9)
A10	35.14 ± 10.619^a (24)	32.17 ± 9.024^a (27)	37 ± 3.05^a (24)	32 ± 2.90^a (27)	20.69 ± 6.450^a (10)	13.92 ± 2.822^a (10)	20 ± 0.77 (10)	$< 1 \pm$ NE (10)
A11	33.85 ± 8.741^a (30)	36.69 ± 8.621^a (30)	37 ± 1.36^a (30)	32 ± 2.71^b (30)	22.33 ± 9.345^a (30)	16.12 ± 3.362^a (30)	21 ± 1.10 (30)	$< 1 \pm$ NE (30)
A12	36.73 ± 11.240^a (28)	36.39 ± 12.436^a (30)	41 ± 1.74^a (28)	30 ± 2.68^b (30)	21.18 ± 5.599^a (30)	15.79 ± 2.606^b (30)	22 ± 0.48 (30)	$< 1 \pm$ NE (30)

Table 3
Median lethal times (LT_{50}, days) and dry weight (mg) of *Osmia bicornis* females and males from the F2 generation (developed in a nonagricultural (NA) landscape) exposed topically to Dursban 480 EC or 0.01% Triton (control). The same lowercase letter indicates no significant differences (at $p \geq 0.01$ after applying the Bonferroni correction for multiple comparisons) in survival (pairwise comparison of survival curves, log-rank test) between exposed and control bees within the nest. Number of bees used is indicated in brackets.

Nest ID	Mean dry weight of females [mg]		LT50 ± SE [days] for females		Mean dry weight of males [mg]	LT50 ± SE [days] for males
	Control (Triton)	Exposed (Dursban)	Control (Triton)	Exposed (Dursban)	Control (Triton)	Control (Triton)
N1	47.88 ± 11.743 (10)	-	20 ± 0.48 (10)	-	17.17 ± 4.955 (21)	19 ± 5.86 (21)
N2	50.18 ± 15.560[a] (30)	42.07 ± 12.288[a] (30)	55 ± 0.61[a] (30)	55 ± 3.22[a] (30)	25.22 ± 5.084 (29)	19 ± 0.29 (29)
N3	37.22 ± 12.674[a] (17)	35.71 ± 13.323[a] (18)	68 ± 2.06[a] (17)	65 ± 2.83[a] (18)	20.21 ± 4.444 (20)	19 ± 1.33 (20)
N5	44.51 ± 10.301 (10)	-	20 ± 0.31 (10)	-	24.16 ± 1.685 (5)	19 ± 3.29 (5)
N6	32.08 ± 13.649 (12)	-	58 ± 2.56 (12)	-	18.00 ± 2.142 (3)	21 ± 11.43 (3)
N7	43.62 ± 9.458[a] (30)	44.29 ± 8.853[a] (28)	64 ± 3.19[a] (30)	64 ± 5.94[a] (28)	23.76 ± 5.331 (30)	19 ± 1.79 (30)
N8	44.26 ± 9.792[a] (30)	43.76 ± 7.902[a] (30)	61 ± 4.68[a] (30)	57 ± 3.19[a] (30)	21.60 ± 5.616 (30)	19 ± 0.79 (30)
N12	37.19 ± 10.974[a] (10)	36.95 ± 7.487[a] (10)	55 ± 1.58[a] (10)	45 ± 8.70[a] (10)	23.52 ± 9.965 (5)	20 ± 7.67 (5)

Institute of Plant Protection, National Research Institute, Białystok, Poland) using a GC–MS/MS technique with LOD = 0.001 μg/ml. The measured concentration of chlorpyrifos was 0.197 μg/μl.

Altogether, four tests toward sensitivity to insecticide were conducted on generation F1: two tests were run with females and two with males, and the number of bees per treatment depended on bee availability (Table 2). Females and males originating from all 12 nests were tested for sensitivity to Dursban 480 EC, but in the F2 generation, only the test toward the sensitivity of females from 5 nests to Dursban 480 EC was conducted due to the generally much lower number of bees available in this generation (Table 1) in the three nests (N1, N5 and N6). Test groups of the F2 females consisted of 10–30 adult bees per nest in both treatments (i.e., with the insecticide and control) depending on availability (Table 3). Moreover, all males from the F2 generation were given only 0.01% Triton X-100 and constituted a control group for checking the survival of F2 males (Table 3). Control bees were included in the study as the untreated bees originating from landscapes with different farming intensity could simply differ in survival rate.

The bees (at least 3 days old; Robinson et al., 2017) were treated individually by topical application of 1 μl of the test solution (either insecticide solution or 0.01% Triton X-100 solution) on the dorsal thorax using a Hamilton microsyringe with a dispenser (Fig. S2). Approximately one hour before treatment, bees were taken out of the cages, placed in glass Petri dishes of 12 cm diameter (maximum 10 bees/dish, 3 dishes/treatment/sex) and placed at 4 °C for approximately 20 min to limit their mobility and ensure proper pesticide application (i.e., prevent the bees from spreading the solution to the neck or wing hinges). The treated bees were then transferred to plastic cages (bees from 3 dishes per box of size 30 ×19.5 ×20.5 cm, Fig. S2) for group housing and moved to the climatic chamber (20 ± 2 °C, 60 ± 5% RH, 16:8 L:D). The bees were fed *ad libitum* with 33% (w/w) sucrose solution placed in Eppendorf tubes (as described above), and their survival was recorded daily until the death of the last bee. The dead bees were consecutively removed from the boxes and frozen in 2-ml Eppendorf tubes at − 20 °C until they were dried at 105 °C for 24 h and weighed to the nearest 0.1 mg.

2.4. Data analysis

The landscape units (Section 2.1) excluding oilseed rape coverage (ORC, treated as a separate explanatory variable) used to characterize the local landscape structure around each nest located in the agricultural (A) landscape were reduced to two factors (FA1, FA2) using factor analysis. Factor analysis with quartimax rotation was computed on standardized units separately for 500 m and 1000 m buffers. Additionally, the local habitat within the 500 and 1000 m buffers was described by the Landscape Diversity Index (LDI) calculated as exp(H'), where H' is the Shannon–Wiener diversity index (Jost, 2007, 2006), using those landscape units that are expected to be functionally relevant for the red mason bee (i.e., vegwat, veginf, bush, for, mead, orch, flo; Table S2) (Fahrig et al., 2011; Mikołajczyk et al., 2021). The local landscape structure around the nests located in the nonagricultural (N) landscape was characterized by the dominance of natural elements – meadows and forests, as most of the other units were not present in the N landscape.

The following life-history traits were measured for the F1 and F2 generations of bees for each nest and were included in the statistical analysis: number of provisioned cells produced, number of cocoons produced, average cocoon weight (data truncated by removing 1% of the minimum weight of cocoons to eliminate empty cocoons, [mg]), adult emergence rate [%], average time to emergence (mean number of days required to emerge from cocoons for females and males separately, after transferring cocoons to 4 °C, [days]) and sex ratio (Female:Male, F:M). Moreover, the sensitivity of bees to Dursban 480 EC was expressed for each nest as the median lethal time (LT_{50}), estimated using Kaplan–Meier survival analysis. The statistical significance of the relationships between all measures of life history traits, including the sensitivity to insecticide (LT_{50}), of bees from each generation (F1 or F2) and the landscape variables (ORC, scores of FA1 and FA2, LDI) was tested with multiple regression analysis separately for 500 m and 1000 m buffers. The regression models were estimated using both nonstandardized and standardized landscape variables. The models estimated using nonstandardized variables can be used as predictors, while standardization allows for direct comparison of the importance (strength) of individual variables. After running the initial model, a backward stepwise selection procedure was used to remove nonsignificant variables, starting with those with the highest p values until only variables with $p \leq 0.05$ remained in the model, and the normal distribution of residuals was formally tested using the Shapiro-Wilk test. The traits expressed as percentage (emergence rate) or proportion (F:M) were transformed using arc sine of the square root transformation (Zar, 1999). Additionally, the proportion of meadows and forests [%] describing the local landscape around the nests (for 500 m and 1000 m separately) in the nonagricultural (N) landscape was used as an explanatory variable for all measures of the life history traits of *O. bicornis* from the F2 generation. In that way, we were able to test not only for the effect of parent origin (i.e., parents nesting along the

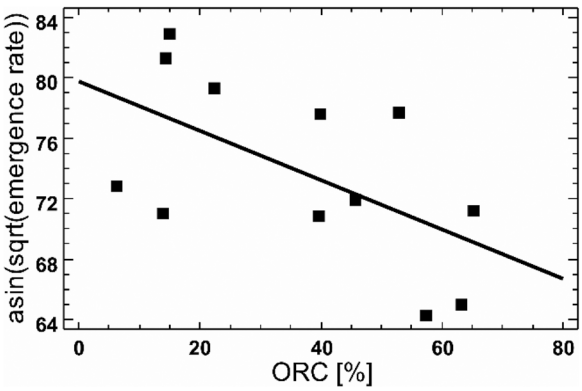


Fig. 2. Results of the multiple regression analysis for the 500 m buffer: negative effect of oilseed rape coverage, ORC ($p = 0.049$, $R^2 = 33.3\%$) on the emergence rate of the F1 generation of *Osmia bicornis* from the 12 nests located in agricultural landscape; arc sine of the square root transformation of emergence rate data was used.

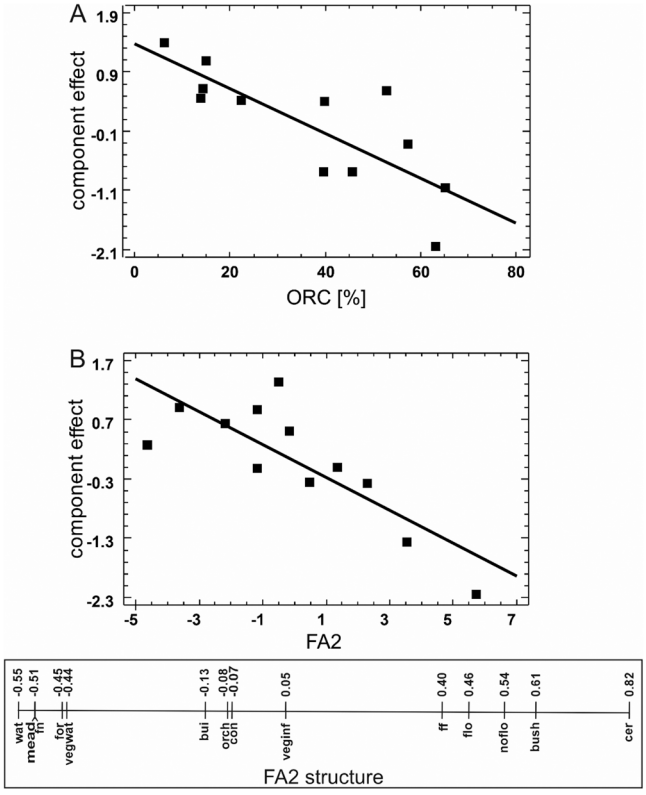


Fig. 3. Results of the multiple regression analysis for the 500 m buffer: negative effects of (A) oilseed rape coverage, ORC ($p = 0.007$) and (B) FA2 ($p = 0.006$) on the average emergence time of *Osmia bicornis* females from the F1 generation originating from 12 nests located in the agricultural landscape. The overall model including both variables was significant at $p = 0.009$ and explained 64.8% of the variability. The line shows the relative change in the predicted values of average emergence time of F1 females that occurs when changing (A) ORC or (B) FA2 over their observed ranges. Each point (site) is then plotted by adding its residuals to a line. Note that the values on the y-axis are the residuals of the part of the model explained by the other significant variable. The bottom additional scale (FA2 structure) shows the variable scores for 14 landscape units describing sites (vegwat - vegetation by water bodies; wat - water bodies, con - concrete and infrastructure; veginf - vegetation by infrastructure; bush - bushes; for - forests; bui - buildings; mead - meadows; orch - orchards; cer - cereals; noflo - nonflowering crops; flo - flowering crops; ff - field-to-field borders; fn - field-to-natural borders; *see* Table S2 *for a full description of the landscape units*) spread on the unitless FA2 axis.

gradient of oilseed rape coverage in landscape A) on the life history traits of their F1 and F2 offspring but also for the possible direct influence of the N landscape on F2 bees.

Survival curves of bees from the F1 generation, either treated with the insecticide or the control, were compared between nests ($p \leq 0.05$, log-rank test), separately for males and females. Pairwise comparisons of survival curves of F1 generation bees between insecticide-treated and control bees were performed for each nest separately using the log-rank test, and the dry weight of bees treated with insecticide versus controls was compared within each nest using a t test (Table 2 and Table 3) with Bonferroni correction for multiple comparisons. Analysis of survival curves and dry weights of bees from the F2 generation were performed similarly but for fewer nests and fewer bees per nest (Table 3).

Data analysis was carried out with the Statgraphics Centurion program (Statgraphics Technologies Inc.) version 18.1.13.

3. Results

3.1. Local landscape characteristics of the study sites

Factor analysis showed the presence of two main gradients of environmental variables among the study sites. In the 500 m buffer, the first factor (FA1) explained 32.4% of the total variability in local landscape characteristics and characterized the dataset according to features related to built-up areas (i.e., concreate, buildings but also vegetation close to infrastructure and orchards) as confronted with seminatural landscape features such as water and vegetation close to water, whereas FA2 explained 21.0% of the total variance and captured the prevalence of "arable lands" features (i.e., cereals and nonflowering and flowering crops but also bushes), as confronted with landscape naturalness (i.e., water bodies and vegetation nearby, meadows, forests, and the length of borders between fields and natural habitats) (Table S3 and Fig. S3A). In the 1000 m buffer, factors FA1 and FA2 explained 29.0% and 27.1% of the total variance, respectively, but this time it was FA1 that captured the prevalence of "arable lands" features (nonflowering crops, flowering crops and cereals but also orchards), as confronted with the length of borders between fields and natural habitats, forests and meadows), whereas FA2 differentiated between sites with a pronounced build-up

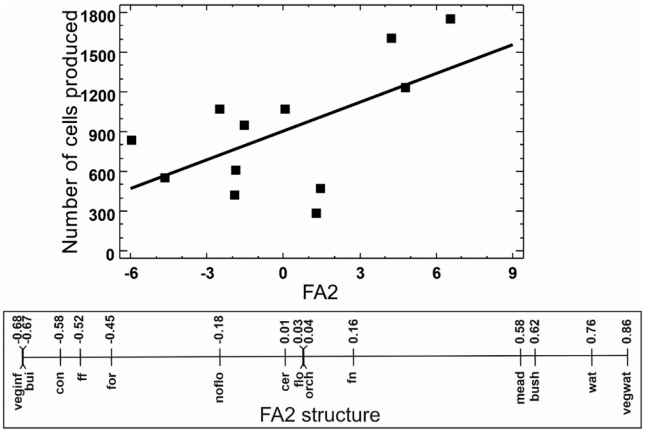


Fig. 4. Results of the multiple regression analysis for the 1000 m buffer: positive effect of FA2 ($p = 0.041$, $R^2 = 35.4\%$) on the total number of cells produced by the parental generation of *Osmia bicornis* females in 12 nests located in the agricultural landscape. The bottom additional scale (FA2 structure) shows the variable scores for 14 landscape units describing sites (vegwat - vegetation by water bodies; wat - water bodies, con - concrete and infrastructure; veginf - vegetation by infrastructure; bush - bushes; for - forests; bui - buildings; mead - meadows; orch - orchards; cer - cereals; noflo - nonflowering crops; flo - flowering crops; ff - field-to-field borders; fn - field-to-natural borders; *see* Table S2 *for a full description of the landscape units*) spread on the unitless FA2 axis.

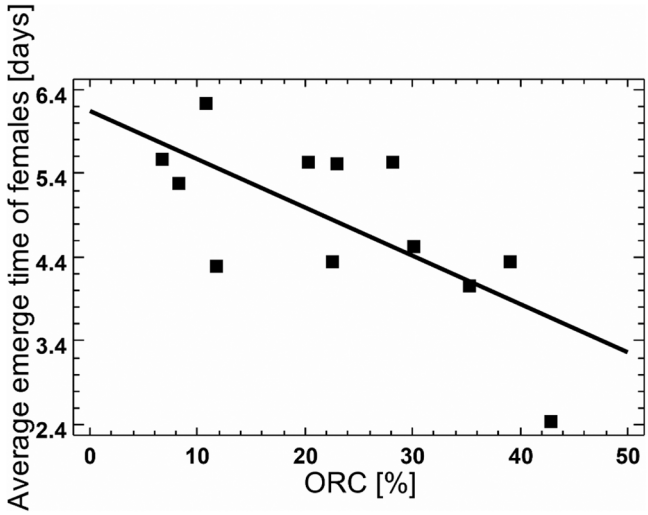


Fig. 5. Results of the multiple regression analysis for the 1000 m buffer: negative effect of oilseed rape coverage, ORC ($p = 0.012$, $R^2 = 48.2\%$) on the average emergence time of *Osmia bicornis* females from the F1 generation originating from 12 nests located in the agricultural landscape.

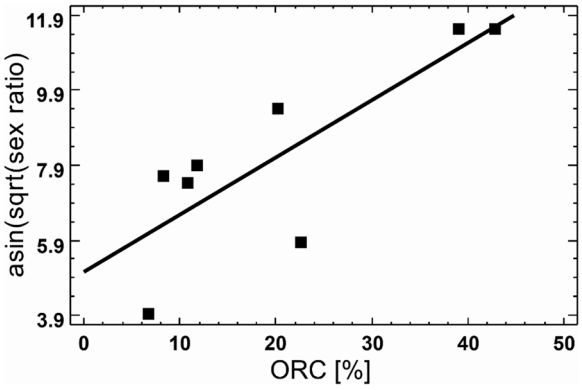


Fig. 6. Results of the multiple regression analysis for the 1000 m buffer: positive effect of oilseed rape coverage, ORC ($p = 0.015$, $R^2 = 65.4\%$) on the sex ratio of bees from the F2 generation., i.e., developed from F1 parents transferred as cocoons to nonagricultural landscapes; arc sine of the square root transformation of sex ratio (Female:Male) data was used.

character (buildings, concreate and vegetation by infrastructure) and those dominated by water bodies and surrounding vegetation, bushes and meadows (Table S3 and Fig. S3B). Thus, the obtained FA1 for the 1000 m buffer and FA2 for the 500 m buffer captured almost the same landscape units, which scored similarly: "arable lands" features (i.e., cereals nonflowering and flowering crops but also bushes and the length of borders between fields) scored high, whereas "landscape naturalness" (meadows, forests, and the length of borders between fields and natural habitats) scored low on those axes. However, FA2 for buffer 1000 m and FA1 for 500 m buffer were inversed: in general, landscape units characteristic for built-up areas (concreate, buildings but also vegetation close to infrastructure) that scored high on FA1 for 500 m buffer, scored low on FA2 for 1000 m and, at the same time, those scored low on FA1 for 500 m (water and vegetation by water) scored high for FA2 for 1000 m. Only some units (e.g., orchards) shifted their position on the FA1 and FA2 factors, scoring higher either in "built-up areas" (FA1, buffer 500 m) or "arable lands" (FA1, buffer 1000 m).

The landscape diversity index (LDI) ranged from 1.21 to 2.55 for the 500 m buffers and from 1.86 to 3.24 for the 1000 m buffers in the agricultural landscape (Table S1). The area around the nest was covered by oilseed rape in 6.4–65.3% and 6.7–42.9% in the 500 m and 1000 m buffers, respectively (Table S1). All sites located in the nonagricultural (N) landscape were covered with meadows and forest in 91.4–98.2% and 92.7–98.0% in the 500 m and 1000 m buffers, respectively (Table S1).

3.2. *Effect of oilseed rape coverage (ORC) and other landscape characteristics on the life history parameters of Osmia bicornis of different generations*

The total number of cells built by *O. bicornis* in 2019 ranged from 284 to 1749, and the number of cells in half of the nests used for population parameter analysis ranged from 56 to 835. At the same time, nest A9 was characterized by the smallest (N = 48), and nest A11 was characterized by the highest (N = 675) number of cocoons (Table 1).

The cocoons from the A7 nest were the heaviest (95.9 ± 31.64 mg), and those from the A4 nest were the lightest (82.7 ± 28.19 mg) (Table 1). Between 81.1% and 98.5% of bees emerged from cocoons (generation F1), and between 29.8% and 50.0% of emerged adults were females (Table 1). It took, on average, between 1.0 ± 0.76 and 1.7 ± 1.74 days for males and between 2.4 ± 2.68 and 6.2 ± 1.22 days for females to emerge after transferring cocoons to 20 °C (Table 1).

For unknown reasons, F1 females that emerged from cocoons originating from the A11 nest did not build their N11 nest in a nonagricultural landscape in spring 2020. In the remaining 8 nests, between 37 (nest N6) and 289 (nest N7) cells with 17–235 cocoons were built. The cocoons from the N7 nest were the heaviest (126.6 ± 34.59 mg), and those from the N5 nest were the lightest (78.0 ± 33.20 mg). Only 28.6% of F2 bees emerged from cocoons in nest N5, and in the other nests, the emergence rate was between 63.3% and 96.6% with a sex ratio toward females (i.e., 51.0–80.0% of emerged bees were females). On average, it took 2.8 ± 0.40–3.7 ± 0.92 days for F2 males and 7.4 ± 1.30–8.3 ± 2.10 days for F2 females to emerge from cocoons after transferring cocoons from overwintering at 4 °C to 20 °C (Table 1).

The parameters of multiple regression models for all studied life-history traits on standardized variables (parameter β allowing for comparisons of model estimates) and nonstandardized (parameter b) variables are presented for the 500 and 1000 m buffers for the F1 and F2 generations in Tables S4 and S5. Below, only significant ($p \leq 0.05$) relationships are presented in more detail.

For generation F1, multiple regression analysis for the emergence rate showed a significant negative relationship with ORC for the 500 m buffer ($p = 0.049$, $R^2 = 33\%$; Fig. 2). A significant negative relationship for the 500 m buffer was also found between the average time to emergence of females and both ORC ($p = 0.007$) and FA2 ($p = 0.006$) (Fig. 3AB); the model including those two explanatory variables was significant at $p = 0.009$ and explained 65% of the variance in the average time to emergence of females (see Table S4 for β parameters). This indicates that the average time to emergence of females decreased not only at sites with high oilseed rape coverage but also at those dominated by cereals and nonflowering crops and bushes. In the 1000 m buffer, the total number of cells produced was positively related to FA2 ($p = 0.041$, $R^2 = 35\%$), showing that the number of cells produced increased with a high proportion of vegetation by water, bushes and meadows and decreased in built-up areas and with agricultural land fragmentation (Fig. 4). Moreover, the average time to emergence of females decreased with ORC ($p = 0.012$, $R^2 = 48\%$) (Fig. 5) in the 1000 m buffer.

For generation F2, the only significant relationship was found between the sex ratio and ORC ($p = 0.015$, $R^2 = 65\%$) for the 1000 m buffer: the proportion of females in newly emerged adults increased with increasing oilseed rape coverage in F1 habitats (Fig. 6).

None of the studied life-history traits of bees from the F2 generation were significantly related to the proportion of meadows and forests around the nests in the N landscape for either the 500 m buffer or the 1000 m buffer.

3.3. Sensitivity of bees toward Dursban 480 EC

Both Dursban-treated (0.2 RAC) ($p = 0.0009$) and control ($p = 0.0006$) females from generation F1 differed significantly between nests in their survival rates ($LT_{50\,s}$ from 29 ± 3.4 to 42 ± 1.4 days and from 35 ± 1.4 to 45 ± 1.6 days, respectively; Table 2). Pairwise comparisons of survival curves within each nest separately showed significant differences between control and Dursban-treated females for four (A5, A7, A11, A12) out of 12 nests with better survival of controls ($p \leq 0.004$; Table 2). These differences were not due to differences in dry body weight, which was similar for Dursban-treated and control females in all 12 nests ($p \geq 0.3$; Table 2).

All F1 males exposed to 0.25 RAC of Dursban died within one day, so only control males were compared between nests showing significant differences in survival curves ($p \leq 0.0001$), with the lowest LT_{50} for the A6 nest (18 ± 0.4 days) and the highest LT_{50} for the A3 nest (27 ± 0.7 days; Table 2). The short lifetime (LT_{50} <1 day) of Dursban-treated males was most likely the reason for significantly lower dry body weight than in control males, which was observed for 6 (A2, A3, A6, A7, A8 and A12) out of 12 nests ($p \leq 0.0001$): Dursban-treated males did not live long enough to gain weight.

No differences between nests were found in the survival of Dursban-treated F2 females from five available nests ($p = 0.2$), but control F2 females originating from eight nests differed in their survival ($p \leq 0.001$), with the lowest $LT_{50\,s}$ of 20 days found for nests N1 and N5 and the highest $LT_{50\,s}$ of 68 days found for nest N3. In contrast to F1 females, no effect of Dursban 480 EC on the survival of F2 females was found for any out of 5 nests available for running pairwise comparison ($p = 0.5$), and Dursban-treated and control F2 females from those 5 nests did not differ in terms of their dry body weight (Table 3). In terms of F2 males, only control males were available for testing for differences in survival between nests, and significant differences in survival curves between nests ($p = 0.03$) were found with $LT_{50\,s}$ between 19 and 21 days (Table 3).

In generation F1, a significant negative relationship was found between the $LT_{50\,s}$ of Dursban-treated females and ORC for the 500 m buffer ($p = 0.009$, $R^2 = 51\%$) (Fig. S4A). A similar relationship was found for the 1000 m buffer, but apart from ORC ($p = 0.0006$), LDI ($p = 0.01$) also negatively affected the LT_{50} of Dursban-treated females ($p = 0.001$, $R^2 = 77\%$) (Fig. S4BC, Table S5). Multiple regression analysis for the $LT_{50\,s}$ of control F1 males showed a significant negative relationship with ORC ($p = 0.02$) and FA2 ($p = 0.02$) for the 500 m buffer (Fig. S5AB); the model including both explanatory variables was significant at $p = 0.025$, $R^2 = 56\%$. Moreover, ORC ($p = 0.03$) and FA1 ($p = 0.04$) negatively affected the LT_{50} of control F1 males in a 1000 m buffer (model significant at $p = 0.01$, $R^2 = 63\%$; Fig. S5CD). Although the survival of control F1 males was negatively related to FA2 for the 500 m buffer and to FA1 for the 1000 m buffer, the conclusion of these results is similar, regardless of the buffer radius: the survival of control F1 males was better at sites with a high proportion of natural habitats (i.e., forests and meadows) and large length of borders between fields and natural habitats and decreased at sites with a high proportion of cereals and nonflowering crops.

For control F2 females, a negative relationship was found between LT_{50} and both ORC ($p = 0.033$) and LDI in the F1 generation habitat ($p = 0.004$), and an opposite relationship with FA1 ($p = 0.011$) was found, only for 500 buffer (Fig. S6ABC). The model including all explanatory variables was significant at $p = 0.012$ and explained 92% of the variance in LT_{50} (Table S4). Additionally, a significant positive relationship was found between the LT_{50} of control males and both ORC ($p = 0.035$) and FA2 ($p = 0.04$) for the 500 m buffer in the F1 generation habitat (model significant at $p = 0.05$, $R^2 = 71\%$; Fig. S7AB). The LT_{50} of control F2 males also increased with ORC ($p = 0.001$) for 1000 m buffer, but FA2 negatively affected the survival of control F2 males ($p = 0.049$) in this buffer (model significant at $p = 0.003$, $R^2 = 90\%$; Fig. S7CD).

No significant relationship was found between the $LT_{50\,s}$ of control males, control females or Dursban-treated females and the proportion of meadows and forests describing areas around the F2 nests (500 m and 1000 m buffer) in the N landscape.

4. Discussion

Although the number of studies on the effects of landscape characteristics on different bee species has increased recently (e.g., Bednarska et al., 2022; Coudrain et al., 2016; Coutinho et al., 2021; Schüepp et al., 2011; Sõber et al., 2020), to the best of our knowledge, this is the first work in which the local landscape structure has been studied for its effect on two subsequent generations of bees. Our previous study already indicted the importance of ORC and landscape structure, but not pollen diversity, for the performance of *Osmia bicornis* (Bednarska et al., 2022). Our main findings in the present study are that the proportion of oilseed rape around the nest affected the bees by decreasing the emergence success, shortening the time to emerge in females from the F1 generation and making them more sensitive toward the Dursban 480 EC insecticide, and shortening the survival time of newly emerged F1 males. However, those effects, together with the effects of landscape structure around the nest, disappeared in the next generation that developed in the areas without agricultural pressure: the only relationship for the F2 generation was found between ORC around the nest in the previous generation and the sex ratio, and, contrary to F1 males, the survival of newly emerged F2 males increased with increasing ORC. Such results indicate that larval development under monoculture farming may have some negative carry-over effects (decreased emergence rate from cocoons and increased sensitivity of adult females to additional stress), but the effect on the next generation was not strong and visible only in shifting the sex ratio toward females. In general, similar relationships between the studied parameters (i.e., average time to emergence for F1 females, sensitivity of newly emerged F1 females to Dursban 480 EC, survival of newly emerged F1 and F2 males) and oilseed rape proportion around nests were found for bees within both studied buffers. This is because the proportion of oilseed rape within a radius of 500 m and 1000 m was highly correlated ($p = 0.0013$, $R^2 = 81\%$). In fact, the only significant relationship with ORC found for the 1000 m buffer but not for the 500 m buffer was for the female rate (positive).

4.1. Carry-over effects of oilseed rape coverage and other landscape characteristics on life history traits and sensitivity to Dursban 480 EC in Osmia bicornis

Although positive effects of oilseed rape on the number of brood cells (Holzschuh et al., 2013; Yourstone et al., 2021) or cocoon weight and weight of newly emerged adult *O. bicornis* (Bednarska et al., 2022) have been previously reported, we did not find similar effects in this study. Here, the total number of cells produced by bees increased on sites with a high proportion of more natural landscape elements (vegetation by water, bushes, meadows) within a radius of 1000 m from the nest and decreased in both built-up areas (areas with buildings, roads, paved areas but also adjacent vegetation characteristic of back-yard terrains in rural areas) and heavily fragmented agricultural areas. Similarly, Persson et al. (2018) did not find significant effects from the area of oilseed rape within 500 m of nests, landscape type (conventional, organic farming or pasture rich) or the length of field borders on the number of brood cells and proportion of female offspring. Our results showed that the more natural the landscape was, the higher the number of brood cells established by mason bees. Such results are in accordance with Park et al. (2015), who showed that natural areas support crop-pollinating insects by providing the necessary resources for foraging, nesting, and population growth that are not available in agricultural fields. Although the emergence success was generally high in all nests (81.1–98.5%), a higher proportion of oilseed rape in the close vicinity of nests of the parental generation of bees resulted in a reduction of emergence success

in their offspring (F1 generation) and shortened time needed for F1 females to emerge. The negative effect of oilseed rape coverage on emergence could have been a result of the pollen quality consumed by F1 bees at their larval stage. Although not tested here, it is known that *Brassicaceae* are a source of *O. bicornis* pollen (Haider et al., 2014; Jauker et al., 2012) and up to 46% on average, but even 100% of *B. napus* pollen was found in some *O. bicornis* nest cells during the oilseed rape blooming period (Teper and Bilinski, 2009). Similarly, our previous study showed that pollen provisions of *O. bicornis* nests collected in agricultural landscapes were dominated by *B. napus* in 9–73%, but the proportion of oilseed rape in the pollen was not related to the ORC in the nest proximity (Bednarska et al., 2022). The relationship between the percentage of oilseed rape in provisions and its coverage was, however, not expected by Bednarska et al. (2022), as, in contrast to this study, bees were allowed to forage over their entire adult life and, because of the exceptionally warm spring, they started building nests long before oilseed rape began to bloom. At the same time, oilseed rape pollen was suggested to be of poor quality for larval development, as individuals raised on pure *B. napus* pollen showed behavioral malfunctions (Dobson et al., 2012). Similarly, Coudrain et al. (2016) suggested that oilseed rape pollen is of poor quality and adversely affects larval development, although it provides an abundant source of nectar for adult bees. The poor quality of oilseed rape pollen was associated with Zn and Cu scarcity (Filipiak and Filipiak, 2020; Filipiak et al., 2022). A high percentage of oilseed rape pollen in provisions may also result in higher pesticide exposure of larvae due to high pesticide use in this crop (Zhang et al., 2017). Indeed, as many as 48 and 34 pesticides were found in oilseed rape pollen and nectar, respectively, some at concentrations exceeding the allowable limits (Wen et al., 2021). As shown by Mokkapati et al. (2021), exposure to insecticide-contaminated pollen may not only cause direct larval mortality but also affect larval development to pupae and imagos. Thus, in order to better understand the impact of ORC on bees, future research should address not only population parameters, but also pollen quality and pesticide contamination.

The only "positive" effect of increasing oilseed rape coverage around the nest for F1 bees was that F1 females emerged faster. Moreover, they emerged faster if they originated from sites dominated by cereals and nonflowering crops in fragmented agricultural landscapes, while the time to emergence increased for females originating from sites with a high proportion of natural landscape elements and when the proportion of field-to-natural borders was high around the nest. A short emergence time and, in turn, less time spent in the nonfeeding life stage should be beneficial for bees, as it reduces preemergence weight loss and increases the postemergence lifespan (Slominski and Burkle, 2019), although we cannot exclude that fast emergence may be at some metabolic cost in terms of weight loss, fat body depletion and associated vigor (Bosch and Kemp, 2000). The effect of ORC on the sex ratio was not visible in the F1 generation, but it was shifted toward females with increasing ORC in the F2 generation. This indicates that F1 females feeding on pollen collected on sites dominated by oilseed rape monoculture at their larval stage produced more female offspring the following year (see further Discussion in *Section 4.2*).

We hypothesized that the increasing oilseed rape coverage around the nest might result in reduced food diversity of pollen and/or increased insecticide risk in that pollen eaten at the larval stage and thus affect the survival of bees in their adult stage and make them more sensitive to additional stressors. Indeed, the adult F1 females that developed in their larval stage in the landscape dominated by the oilseed rape monoculture lived for a shorter amount of time after exposure to the insecticide Dursban 480 EC. It has been previously shown that larval exposure to pesticides has a carry-over effect on adults by shortening the lifespan of lab-reared solitary bees (Anderson and Harmon-Threatt, 2019). Additionally, insecticide exposure directly to foraging adults and via carry-over effects from past exposure reduced the reproduction of *Osmia* bees (Stuligross and Williams, 2021). The control F1 males also survived shorter with increasing proportion of oilseed rape around the nest. Moreover, they survived shorter if they originated from sites dominated by "arable lands" (i.e., cereals and nonflowering crops) than from those dominated by more natural habitats (i.e., forests and meadows) with large length of borders between field and natural habitats. Such results confirm the importance of natural landscape elements for the postemergence lifespan of *O. bicornis* males. Even if the role of males is limited to insemination (they are not involved in the construction of the nest cells and collecting pollen for future offspring, Raw, 1972) and they spend more time in close proximity to the nest, still the better survival of *O. bicornis* males translate into higher fertilization success.

4.2. Maternal effects resulted from exposure of a previous generation of *Osmia bicornis* to increasing oilseed rape coverage around the nest

Previous studies have shown that the availability of resources affects the sex ratio: females invest in female production, which requires larger pollen resources when resources are available (Ivanov, 2006; Splitt et al., 2021b). However, in our study, the shift of the sex ratio toward females with increasing ORC was visible not in the F1 generation but in their offspring, even if those offspring developed in natural landscapes, i.e., without pressure from a possible monotonous and pesticide-contaminated diet and with similar access to floral resources. Thus, although all F1 adult females had similar access to the resources for their offspring in nonagricultural landscapes, those that developed in their larval stage in landscapes dominated by oilseed rape monocultures invested more in female than male offspring. This confirms that the environment at the early stage of mother development had an indirect, delayed impact on subsequent generations. Assuming that it is evolutionarily more beneficial, although more costly, to produce females (Bosch, 2008), shifting offspring sex toward females is the positive effect. Thus, adult bees that developed as larvae in the sites with higher ORC, by investing more in female offspring, increased their fitness.

Carry-over effects resulting from the larval food environment are relatively well documented, especially in insects with complete metamorphosis from larval to adult stages, for which most feeding occurs during the larval stage (De Block and Stoks, 2005). The effects observed in offspring phenotypes, including changes in the sex ratio and insecticide resistance in insects, have been attributed to maternal effects (Mousseau and Dingle, 1991). However, it is often difficult to separate maternal effects from the offspring environment. To do this, we ensured that the F2 offspring developed without any pressure from the agricultural landscape by establishing their parental generation from F1 cocooned adults in a nonagricultural area and letting newly emerged F1 bees originate from all nests located on the ORC gradient to forage for food for their progeny in very similar environments, regardless of their origin. In that way, whatever "effects" were passed by mother bees (F1) onto their offspring should not be the result of differences in pollen provisions (similar for all populations) and nesting materials (identical for all populations) but must result from past exposure of the mothers at their larval stage. We checked whether the F2 generation acquired resistance to pesticides but found no confirmation for this: although F1 females had shorter life spans with increasing oilseed rape coverage around the nests from which they originated, no relationship between $LT_{50\,s}$ of Dursban 480 EC-treated females and ORC around their parent's nests was found for the F2 generation. However, we need to stress here that the limited number of nests was available for testing the effect of Dursban 480 EC on the F2 generation. The negative effect of ORC in the previous generation was only visible in the control (Triton-treated) F2 females, for which survival time decreased with increasing ORC (and at the same time with increasing proportion of build-up areas around parental nests), while the opposite relationship was found for the control F2 males. The control F2 males survived longer if their parents developed in areas with a high proportion of cereals and other nonflowering crops and in highly fragmented agricultural landscapes rather than more natural landscapes. Such result is hard to explain, as the trend was

opposite than those found for survival of F1 control males (i.e., negative relationships between LT_{50} values and both ORC and FA2). We must mention, however, that due to the insufficient number of cocoons in some nests collected from the field with F1 larvae and the loss of one nest that was not inhabited by F1 bees after transferring cocooned adults to the nonagricultural landscape, data for only 8 out of 12 nests were available for the study on the F2 generation, which might affect the obtained results. Moreover, the numbers of F2 males and females were lower than those obtained in the F1 generation. Thus, for multigenerational studies, a larger number of nests/sites with a larger number of cocooned adults is recommended for nest loss due to unpredictable random events.

5. Conclusions

Our study revealed that past exposure to environmental stressors, herein related to the development of larvae in pesticide-treated monocultures, may have carry-over effects at the adult stage and can have even maternal effects across generations. In our study, the carry-over effect of developing in areas with increasing dominance by oilseed rape was visible as the decrease in emergence success and the higher sensitivity of newly emerged females toward insecticide. The across-generation maternal effect was seen as the shift in offspring sex toward females. Carry-over and maternal effects have implications for population persistence in agricultural landscapes and should be included in risk assessment, conservation, and management decisions for pollinators to mitigate the effects of agricultural landscape structure. Understanding the multigenerational effects of mass-flowering crops and landscape complexity in general on the fitness of wild pollinators would promote their maintenance in the long term.

We also showed that multiple generations of *O. bicornis* bees benefit from landscape elements that describe landscape naturalness, i.e., water bodies and vegetation nearby, meadows, forests and the length of borders between fields and natural habitats. These landscape elements, being sources of heterogeneity, should be considered to support beneficial insects in agricultural landscapes, as they positively affected the number of brood cells established by the parental generation of bees, shortened the emergence time of F1 females and increased F1 male survival. The importance of seminatural elements and other permanent landscape features is consistent with previous studies, showing that the loss of seminatural habitats is one of the main drivers of pollinator decline (Ricketts et al., 2008; Tscharntke et al., 2012).

Funding

This study was supported by the National Science Centre, Poland within SONATA 13 (2017/26/D/NZ8/00606) and access to research facilities by the subsidy for scientific activity of the Jagiellonian University (N18/DBS/000003).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

We would like to thank Ryszard Laskowski from the Institute of Environmental Sciences, Jagiellonian University, for constructive comments.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2023.108514.

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SUPPLEMENTARY MATERIALS

Impact of oilseed rape coverage and other agricultural landscape characteristics on two generations of the red mason bee *Osmia bicornis*

Anna Misiewicz[1*], Łukasz Mikołajczyk[1,2], Agnieszka J. Bednarska[1]

[1]Institute of Nature Conservation, Polish Academy of Sciences, A. Mickiewicza 33, 31-120 Kraków, Poland

[2]Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

[*]Corresponding author: Anna Misiewicz, e-mail: misiewicz@iop.krakow.pl

TABLES AND FIGURES

Table S1. Characteristics of the study sites in the close vicinity of *Osmia bicornis* nests (i.e., within 500 and 1000 m radius around each nest, called "buffers") in agricultural (A) and nonagricultural (N) landscapes selected for the field study in 2019 and 2020, respectively; ORC – oilseed rape coverage [%], LDI - Landscape Diversity Index (*see text for more details*).

Agricultural (A) landscape							Non-agricultural (N) landscape				
Nest ID	Agricultural area [%] within 5x5 km around the nest	Natural area [%] within 5x5 km around the nest	ORC [%] in 500 m buffer	ORC [%] in 1000 m buffer	LDI in 500 m buffer	LDI in 1000 m buffer	Nest ID	Agricultural area [%] within 5x5 km around the nest	Natural area [%] within 5x5 km around the nest	Meadows and forests [%] in 500 m buffer	Meadows and forests [%] in 1000 m buffer
A1	88	10	6.35	6.73	3.22	3.2	N1	20	93	98.17	97.99
A2	91	12	13.93	11.83	2.24	3.07	N2	12	93	96.02	97.13
A3	57	44	14.33	8.2	1.94	1.86	N3	22	91	96.88	92.69
A4	81	21	14.98	22.93	2.53	3.14	-	-	-	-	-
A5	71	31	22.48	10.77	2.55	2.8	N5	13	93	95.13	96.07
A6	93	11	39.56	42.87	1.69	2.09	N6	14	89	93.15	95.78
A7	84	17	39.98	20.26	2.53	3.24	N7	20	86	96.07	97.08
A8	80	21	45.73	22.62	1.66	3.08	N8	14	92	96.23	94.76
A9	94	6	52.85	28.16	1.56	2.09	-	-	-	-	-
A10	81	20	57.33	30.15	1.21	2.94	-	-	-	-	-
A11	91	15	63.23	35.27	2.13	2.79	N11	10	97	91.41	95.29
A12	92	12	65.3	39.02	1.52	2.47	N12	23	87	97.1	96.02

Table S2. Description of landscape units used for characterization of buffers around each site in agricultural landscape (1-13) (Mikołajczyk et al. 2021). Two additional landscape characteristics (14-15) were calculated within the buffers around nests.

Landscape unit	Name	Acronym	Description
1	Vegetation by water bodies	vegwat	Encompasses (an arbitrarily chosen if not already mapped) 2 m wide strip of terrain that surrounds lakes, ponds and runs along both sides o streams, brooks, rivers, drainage ditches and hydro-technical channels of a different sort. Because of its peripheral location, this type of vegetation is rarely maintained or cut and seems to remain in a relatively untouched state throughout the whole year.
2	Water bodies	wat	Groups all bodies of water, flowing and standing, of natural and anthropogenic origin.
3	Concrete, asphalt, infrastructure	con	Groups all anthropogenically paved terrains – roads, walkways, paved yards, and other infrastructural objects like pylons, wind turbines, and transmission towers.
4	Vegetation by infrastructure	veginf	Groups vegetation around roads, walkways, yards, and infrastructural objects. This vegetation is often maintained is some way (roads) but might as well stay forsaken (back yards).
5	Bushes	bush	Groups terrains covered with perennial plants, bushes, shrubs, overgrown uncultivated lands but not yet forests. Group gathers also urban parks and cemeteries.
6	Forests	for	Groups terrains covered with trees and underbrush.
7	Buildings	bui	Gathers man-made structures of habitual or industrial character – edifices, houses, factories, warehouses, etc.
8	Meadows	mead	Groups terrains covered by grasslands and meadows, offering an abundance of flowering plants when not maintained.
9	Orchards	orch	Groups terrains with perennial, flowering fruits plantations.
10	Cereals	cer	Gathers agricultural land with anemophilous grasses cultivated for grain. Terrains prone to agricultural treatment (e.g. insecticide spraying).
11	Non-flowering crops	noflo	Groups agricultural land with crops not producing regular flowers or harvested before blooming. Terrains prone to agricultural treatment.
12	Flowering crops	flo	Gathers agricultural land with crops producing flowers. Terrains prone to agricultural treatment.
13	Oilseed rape	oil	Groups agricultural land planted with intensively flowering oilseed rape (*Brassica napus*). Prone to heavy agricultural treatment.
14	Field-to-field borders	ff	Counts the total length of field boundaries and is used as a proxy for average agricultural plot size and land fragmentation.
15	Field-to-natural borders	fn	Counts the total length of boundaries between agricultural land and natural (or semi-natural) habitats and is used as a proxy for potential shelter availability for arthropods in an agricultural landscape.

Table S3. Results of factor analysis (factor scores after quartimax rotation) for 14 landscape units describing sites within 500 m and 1000 m radius around each *Osmia bicornis* nest (called "buffers" in agricultural (A) landscape. *See Table S2 for a full description of the landscape units.*

Acronym variable name	Variable description	500m buffer		1000m buffer	
		Factor score FA1	Factor score FA2	Factor score FA1	Factor score FA2
wegwat	vegetation by water bodies	-0.47	-0.44	-0.07	0.86
wat	water bodies	-0.49	-0.55	-0.17	0.76
con	concrete, asphalt, infrastructure	0.93	-0.07	-0.15	-0.58
veginf	vegetation by infrastructure	0.9	0.05	-0.32	-0.68
bush	bushes	-0.13	0.61	0.45	0.62
for	forests	-0.07	-0.45	-0.68	-0.45
bui	buildings	0.92	-0.13	0.15	-0.67
mead	meadows	0.61	-0.51	-0.54	0.58
orch	orchards	0.91	-0.08	0.8	0.04
cer	cereals	-0.02	0.82	0.55	0.01
noflo	non-flowering crops	-0.18	0.54	0.74	-0.18
flo	flowering crops	-0.16	0.46	0.83	0.03
fn	field to natural borders	-0.22	-0.51	-0.78	0.16
ff dens	density of field to field borders	0.45	0.4	0.32	-0.52
% variability explained		32.38	21.01	29.03	27.14

Table S4. The results of fitting multiple regression analysis for 500 m to describe the relationship between explanatory variable of life history of *Osmia bicornis* from F1 or F2 generation (i.e., total number of provisioned cells, adult emergence rate [%], average time to emergence (mean number of days required to emerge from cocoons for females after transferring cocoons to 4°C, [days]), sex ratio of emerged adults (Female: Male), survival of control males or females and survival of Dursban 480 EC exposed females (expressed as LT_{50}, days)) and four independent variables (oilseed rape coverage (ORC, %), FA1, FA2 and LDI). The values of parameters b, β (for the model on standardized variables) and p for the variables included in the final model, i.e., the model with only significant explanatory variables (at $p \leq 0.05$) after backward stepwise selection are presented together with R^2, R^2_{adj} and p values for the final model.

Generation	Exponatory variable		Independent variable						
			ORC	FA1	FA2	LDI	p value for the model	R^2	R^2_{adj}
F1	Emergence rate [%]	p	0.049				0.049	33.3	26.7
		b	-0.163						
		β	-2.968						
	Average time to emerge for females [day]	p	0.007		0.006		0.009	64.8	57.0
		b	-0.038		-0.278				
		β	-0.688		-0.927				
	LT_{50} for Dursban 480 EC treated females [day]	p	0.009				0.009	50.7	45.8
		b	-0.124						
		β	-2.259						
	LT_{50} for control males [day]	p	0.018		0.016		0.025	55.8	46.0
		b	-0.095		-0.697				
		β	-1.720		-2.323				
F2	LT_{50} for control females [day]	p	0.033	0.011		0.004	0.012	91.9	85.9
		b	-0.750	3.340		-57.545			
		β	-13.149	16.456		-35.086			
	LT_{50} for control males [day]	p	0.035		0.038		0.046	70.8	59.0
		b	0.027		0.170				
		β	0.048		0.591				

Table S5. The results of fitting multiple regression analysis for 1000 m buffer to describe the relationship between explanatory variable of life history of *Osmia bicornis* from F1 or F2 generation (i.e., total number of provisioned cells, adult emergence rate [%], average time to emergence (mean number of days required to emerge from cocoons for females after transferring cocoons to 4°C, [days]), sex ratio of emerged adults (Female: Male), survival of control males or females and survival of Dursban 480 EC exposed females (expressed as LT_{50}, days)) and four independent variables (oilseed rape coverage (ORC, %), FA1, FA2 and LDI). The values of parameters b, β (for the model on standardized variables) and p for the variables included in the final model, i.e., the model with only significant explanatory variables (at $p \leq 0.05$) after backward stepwise selection are presented together with R^2, R^2_{adj} and p values for the final model.

Generation	Exponatory variable		Independent variable						
			ORC	FA1	FA2	LDI	p value for the model	R^2	R^2_{adj}
F1	Total number of provisioned cells	p			0.041		0.041	35.4	29.0
		b			72.624				
		β			242.083				
	Average time to emerge for females [day]	p	0.012				0.012	48.2	43.0
		b	-0.058						
		β	-1.050						
	LT_{50} for Dursban 480 EC treated females [day]	p	0.001			0.010	0.001	77.2	72.2
		b	-0.262			-4.149			
		β	-4.755			-2.583			
	LT_{50} for control males [day]	p	0.027	0.038			0.011	63.4	55.3
		b	-0.122	-0.337					
		β	-2.213	-1.415					
F2	Sex ratio (Female:Male)	p	0.015				0.015	65.4	59.6
		b	0.152						
		β	2.672						
	LT_{50} for control males [day]	p	0.001		0.049		0.003	90.0	86.6
		b	0.057		-0.079				
		β	1.005		-0.274				

Figure S1. (A) The artificial nest made of polystyrene 16 elements (nesting cases), stacked on top of each other. Nesting cases were placed in the box-shaped housing made of durable and weather-resistant polypropylene together with a carton box with *Osmia bicornis* cocoons. To protect of solitary bees against birds or rodents, each nest was equipped with plastic grid (1×1 cm) and attached to a wooden pole at a height of ca. 1 m above the ground. (B) Example of nesting case with eggs laid on the top of pollen provision stored in a walk-in climatic chamber, under changing temperature conditions (ca. two months at 20°C, next two months at 15°C, then a month at 10°C and finally overwintering at 4°C) to breed F1 generation. (C) Example of nesting case with F1 cocoons produced.

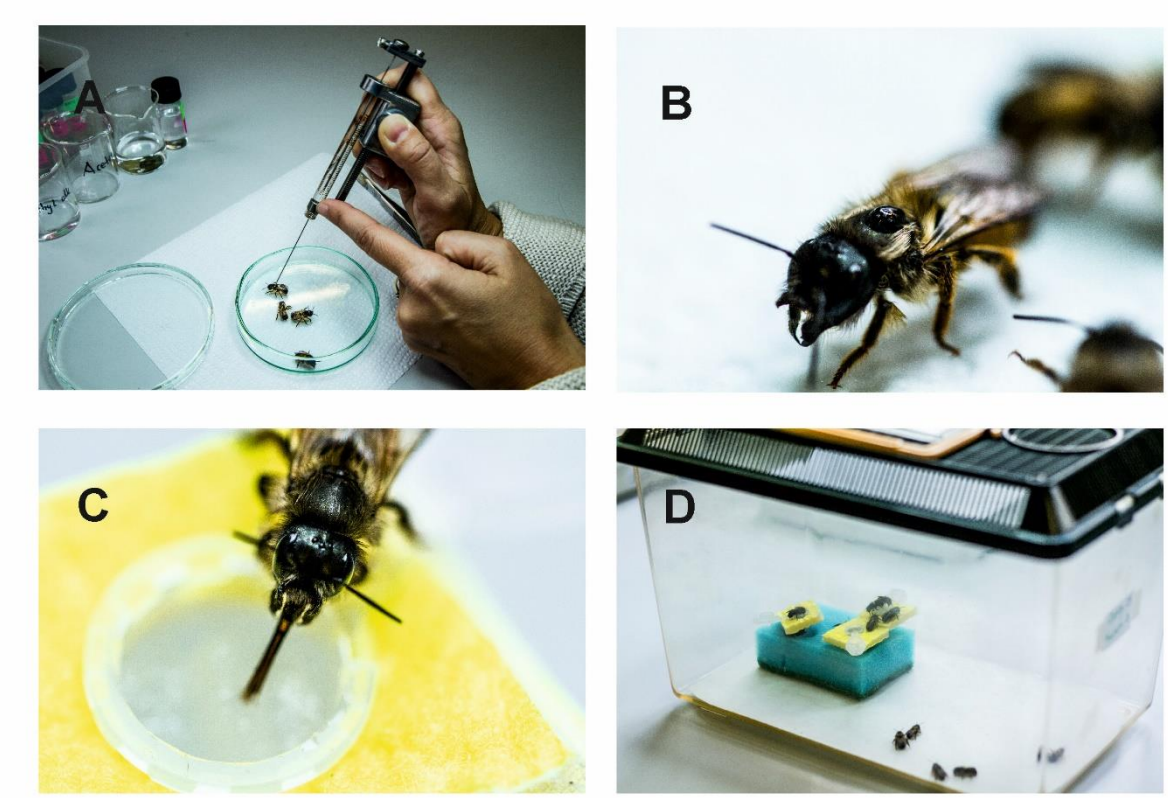

Figure S2. (A, B) Topical application of the treatment solution to bee female on glass Petri dishes using Hamilton micro-syringe; (B) Eppendorf tubes used for feeding bees with sucrose solution 33% (w/w), with cotton wool inside to prevent bees from entering the tubes and with a small square-cut piece of yellow sponge-cloth provided around the tube to attract the bees to the food. (D) Plastic box used for group housing after application of treatment solution.

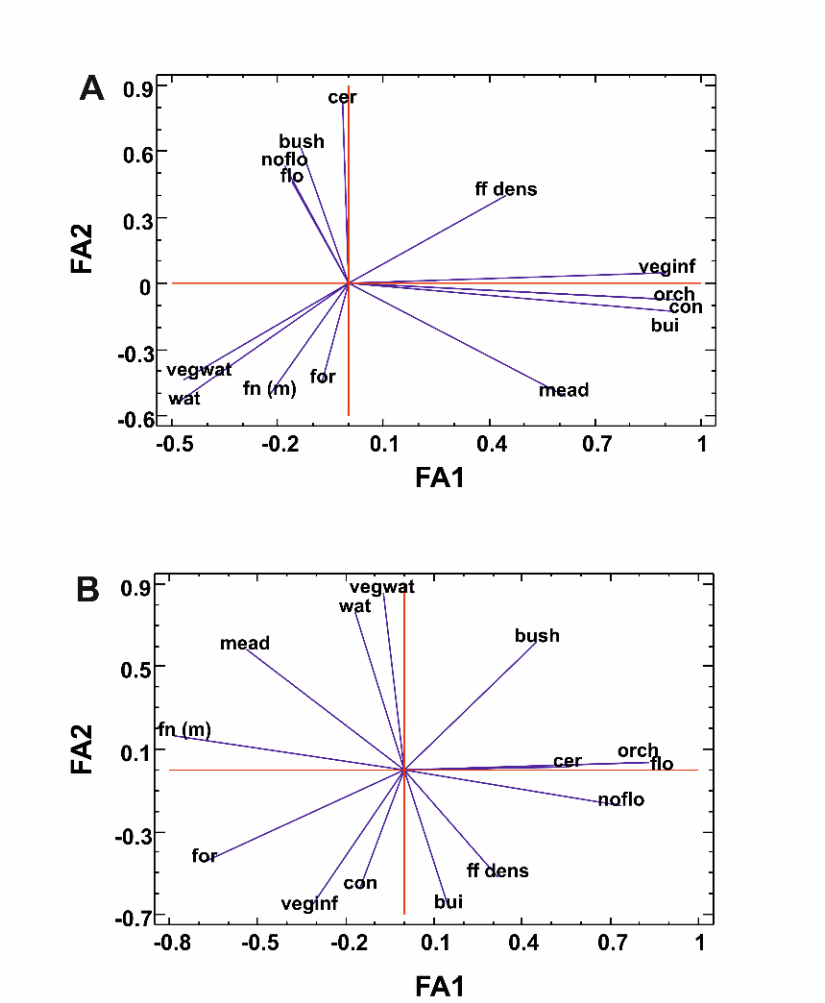

Figure S3. Plots of factors loadings depicting results of Factor Analysis for (A) 500 m buffer and (B) 1000 m buffer with the variable scores plotted over an ordination plane with axes representing the first two FA factors (FA1 and FA2). *Variable names as described in Table S1*.

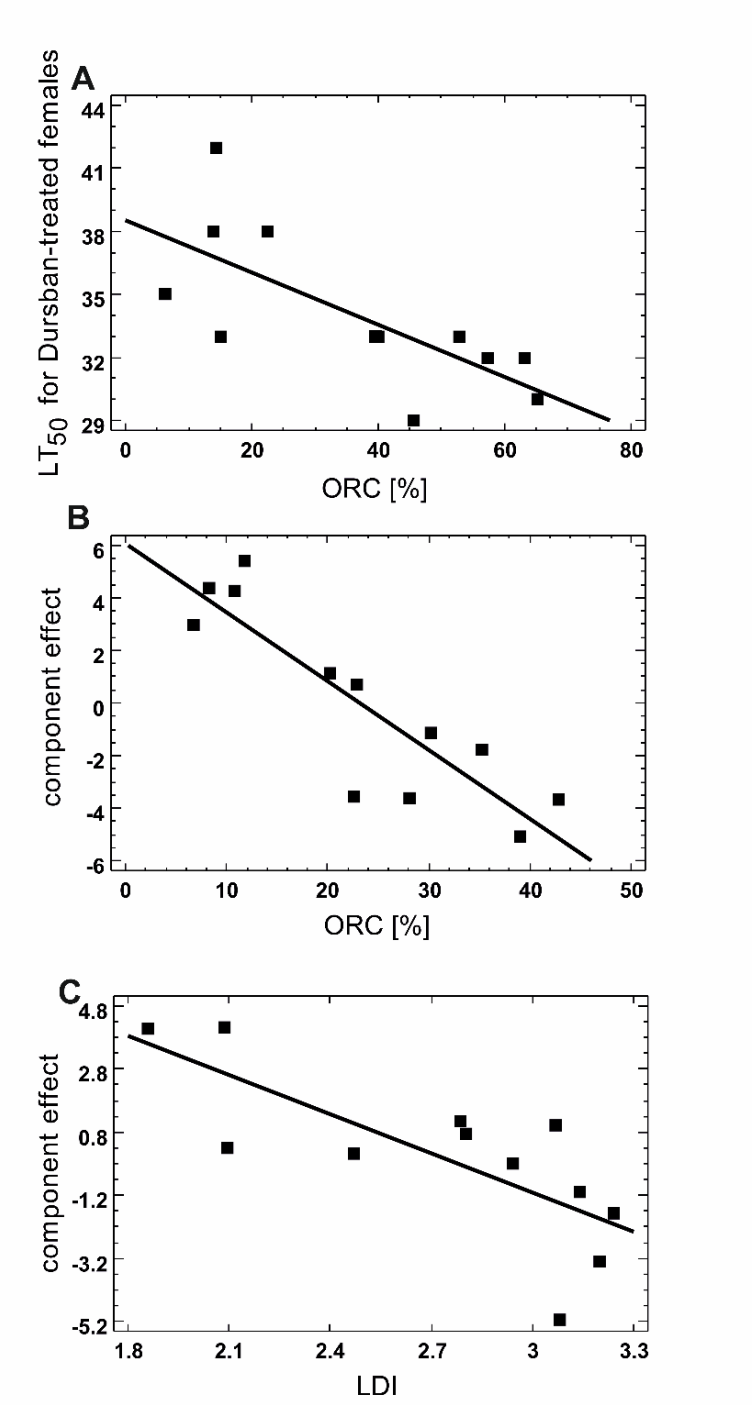

Figure S4. Results of the multiple regression analysis for (A) the 500 m buffer and (B, C) the 1000 m buffer: (A) negative effect of oilseed rape coverage, ORC (p=0.009, R^2=50.7%) on the LT_{50} of Dursban-treated *Osmia bicornis* females from F1 generation (developed in agricultural landscape). In the 1000 m buffer, apart from (B) oilseed rape coverage, ORC (p=0.0006), (C) Landscape Diversity Index, LDI (p=0.0096) affected the LT_{50} of Dursban-treated *Osmia bicornis* females from F1 generation; the overall model including both variables was significant at p=0.001 and explained 77.2% of the variability. The line shows the relative change in the predicted values of the LT_{50} of Dursban-treated F1 females that occurs when changing (A, B) ORC or (C) LDI over their observed ranges. Each point (site) is then plotted by adding its residuals to a line. Note that the values on the y-axis are the residuals of the part of the model explained by the other significant variable.

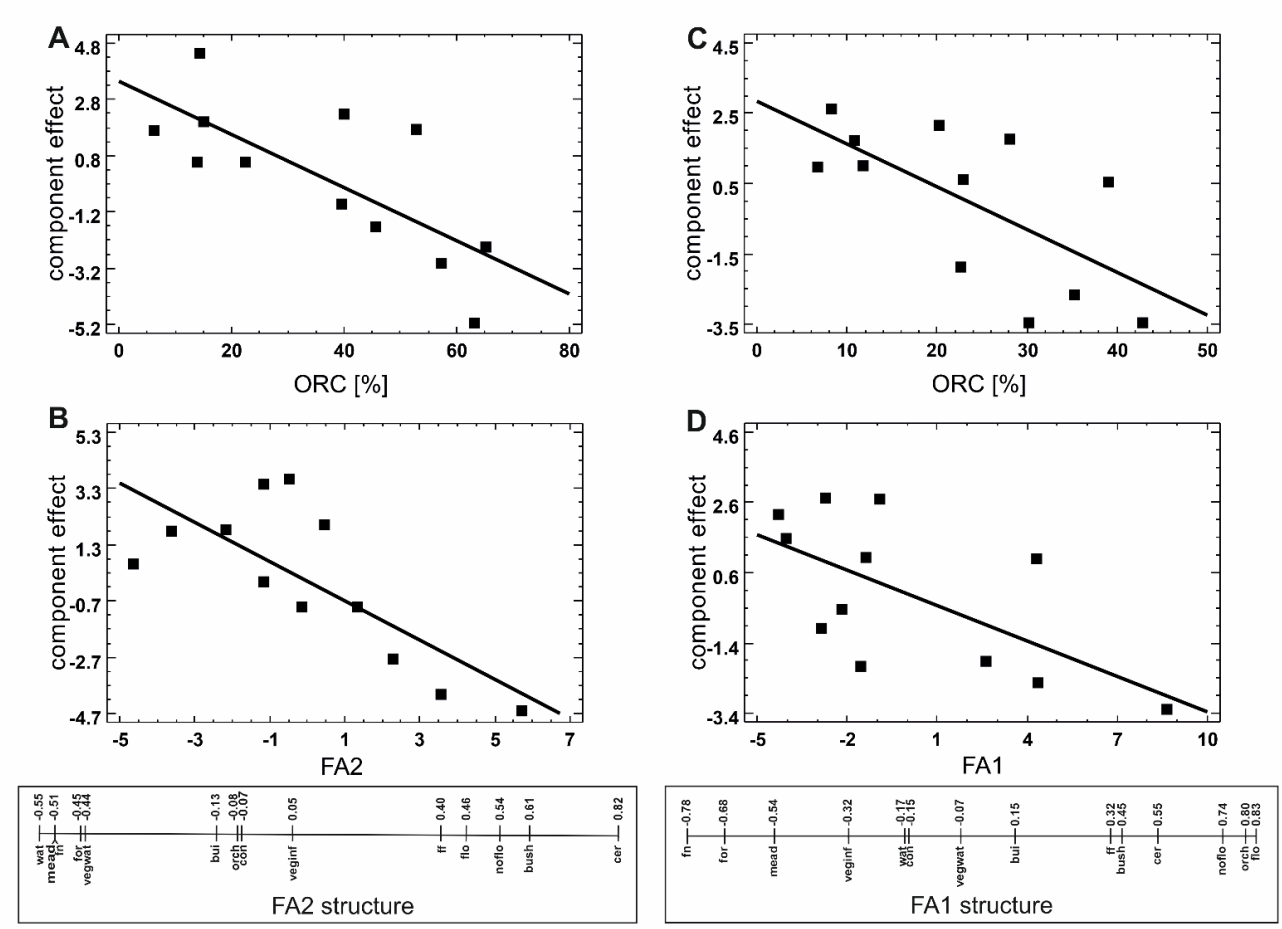

Figure S5. Results of the multiple regression analysis for (A, B) the 500 m buffer and (C, D) the 1000 m buffer: (A) negative effect of oilseed rape coverage, ORC (p=0.02) and (B) FA2 (p=0.02) on the LT_{50}s of control *Osmia bicornis* males from F1 generation (developed in agricultural landscape); the overall model including both explanatory variables was significant at p=0.025, R^2=56%. In the 1000 m buffer, negative effect of (C) oilseed rape coverage, ORC (p=0.03) and (D) FA1 (p=0.04) was found; the overall model including both significant explanatory variables was significant at p=0.01, R^2=63%. The line shows the relative change in the predicted values of the LT_{50}s of control *Osmia bicornis* males from F1 generation that occurs when changing (A, C) ORC, (B) FA2 or (D) FA1 over their observed ranges. Each point (site) is then plotted by adding its residuals to a line. Note that the values on the y-axis are the residuals of the part of the model explained by the other significant variable. The bottom additional scale below Figures B and D (FA2 and FA1 structures, respectively) shows the variable scores for the 14 landscape units describing sites (vegwat - vegetation by water bodies; wat - water bodies, con - concrete and infrastructure; veginf - vegetation by infrastructure; bush - bushes; for - forests; bui - buildings; mead - meadows; orch - orchards; cer - cereals; noflo - nonflowering crops; flo - flowering crops; ff - field-to-field borders; fn - field-to-natural borders; *see Table S2 for a full description of the landscape units*) spread on the unitless FA2 (B) and FA1 (D) axes.

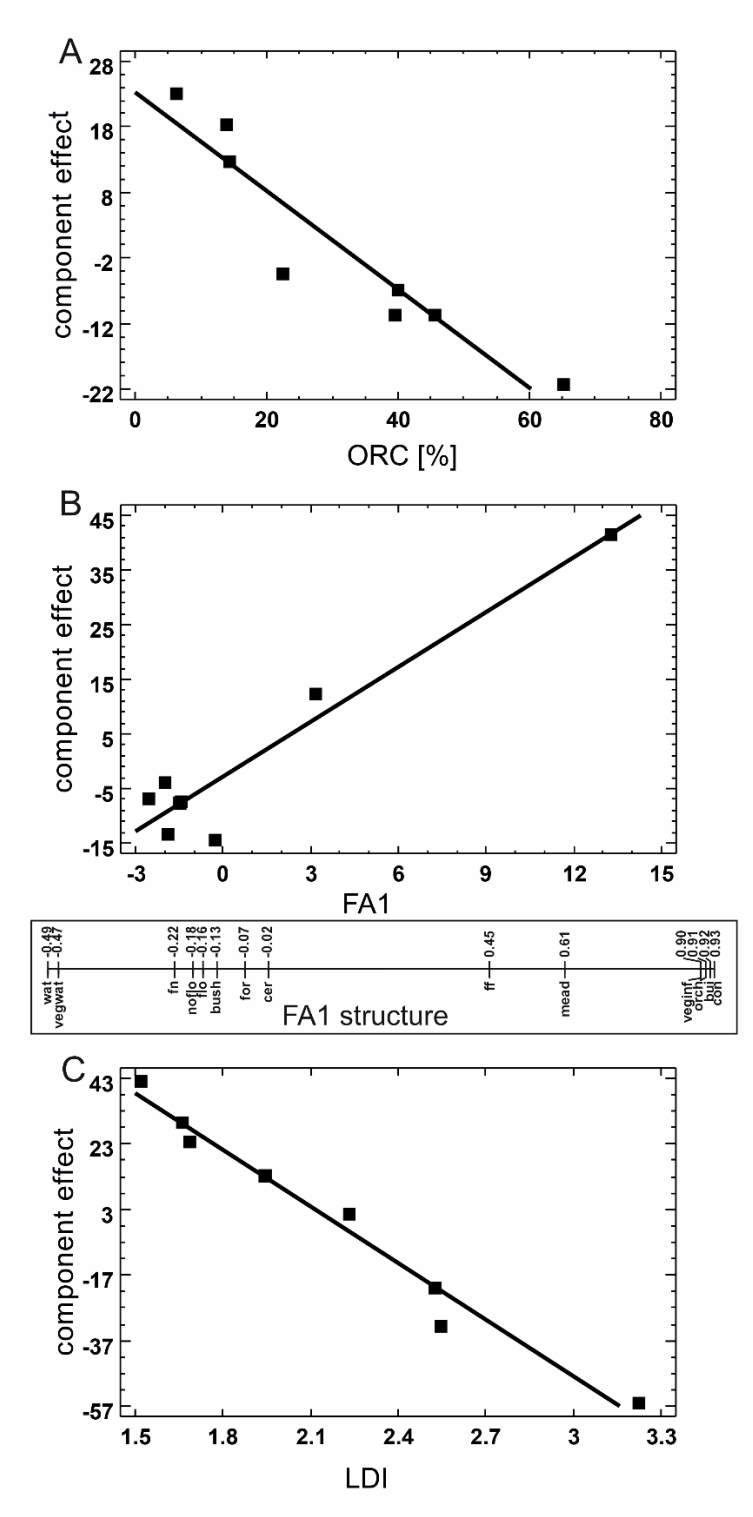

Figure S6. Results of the multiple regression analysis for the 500 m buffer: negative effect of (A) oilseed rape coverage, ORC (p=0.03) and (B) FA1 (p=0.01) on the LT_{50}s of control *Osmia bicornis* females from F2 generation (developed in non-agricultural landscape). The overall model including all variables was significant at p=0.012 and explained 91.9% of the variability. The line shows the relative change in the predicted values of the LT_{50}s of control *Osmia bicornis* females from F2 generation that occurs when changing (A) ORC, (B) FA1 and (C) LDI over their observed ranges. Each point (site) is then plotted by adding its residuals to a line. Note that the values on the y-axis are the residuals of the part of the model explained by the other significant variable. The bottom additional scale below Figure B (FA1 structure) shows the variable scores for the 14 landscape units describing sites (vegwat - vegetation by

water bodies; wat - water bodies, con - concrete and infrastructure; veginf - vegetation by infrastructure; bush - bushes; for - forests; bui - buildings; mead - meadows; orch - orchards; cer - cereals; noflo - nonflowering crops; flo - flowering crops; ff - field-to-field borders; fn - field-to-natural borders; *see Table S2 for a full description of the landscape units*) spread on the unitless FA1 axis.

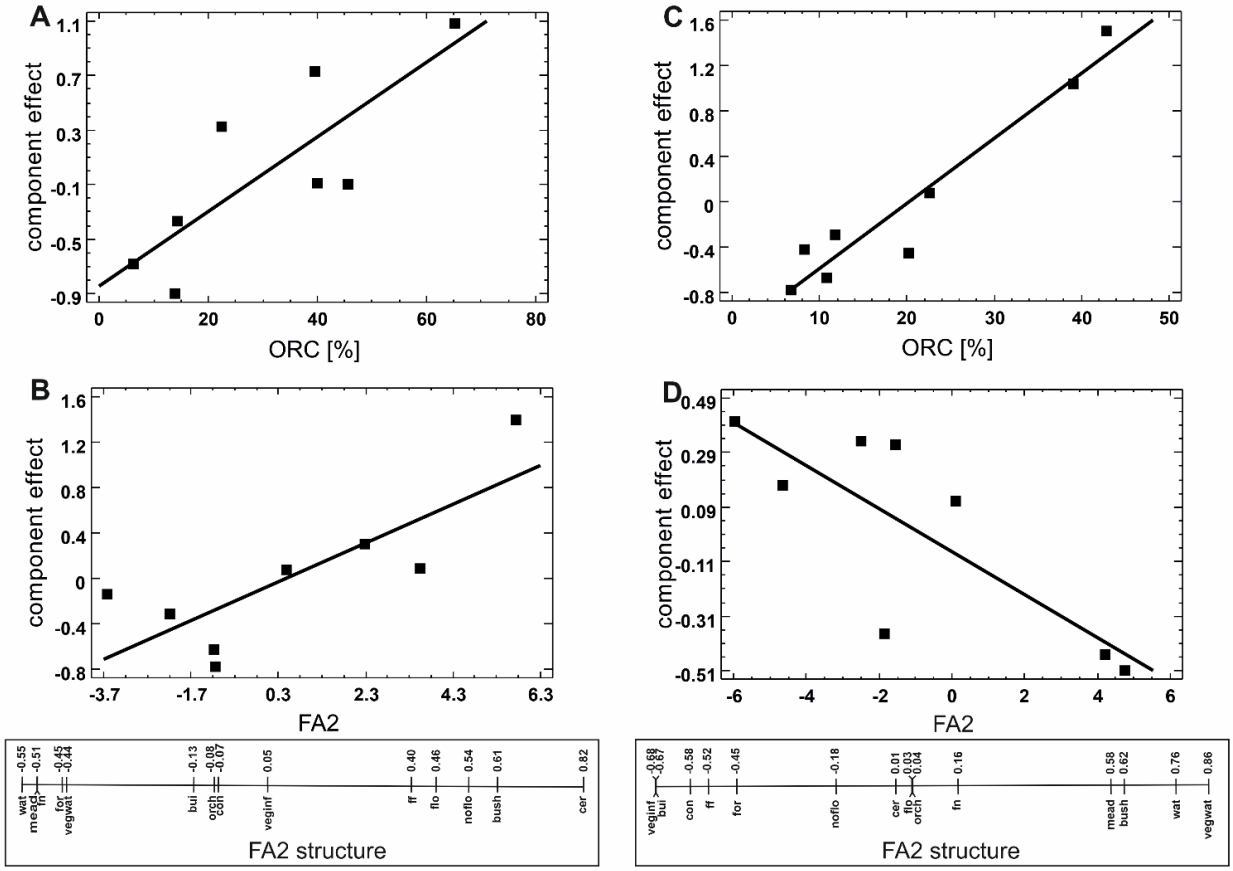

Figure S7. Results of the multiple regression analysis for (A, B) the 500 m buffer and (C, D) the 1000 m buffer: (A) positive effect of oilseed rape coverage, ORC (p=0.035) and (B) FA2 (p=0.038) on the LT_{50}s of control *Osmia bicornis* males from F2 generation (developed in non-agricultural landscape); the overall model including both explanatory variables was significant at p=0.046, R^2=70.8%. In the 1000 m buffer, positive effect of (C) oilseed rape coverage, ORC (p=0.001) and negative effect of (D) FA2 (p=0.049) was found; the overall model including both significant explanatory variables was significant at p=0.003, R^2=90.4%. The line shows the relative change in the predicted values of the LT_{50}s of control *Osmia bicornis* males from F2 generation that occurs when changing (A, C) ORC and (B, D) FA2 over their observed ranges. Each point (site) is then plotted by adding its residuals to a line. Note that the values on the y-axis are the residuals of the part of the model explained by the other significant variable. The bottom additional scale below Figures B and D (FA2 structures) shows the variable scores for the 14 landscape units describing sites (vegwat - vegetation by water bodies; wat - water bodies, con - concrete and infrastructure; veginf - vegetation by infrastructure; bush - bushes; for - forests; bui - buildings; mead - meadows; orch - orchards; cer - cereals; noflo - nonflowering crops; flo - flowering crops; ff - field-to-field borders; fn - field-to-natural borders; *see Table S2 for a full description of the landscape units*) spread on the unitless FA2 axes.

ARTYKUŁ II

Misiewicz, A., Mikołajczyk, Ł., Bednarska, A.J., 2023. Floral resources, energetic value and pesticide residues in provisions collected by *Osmia bicornis* along a gradient of oilseed rape coverage. Scientific Reports 13, 13372. https://doi.org/10.1038/s41598-023-39950-5 (IF = 4,6, 140 pkt MNiSW).

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Floral resources, energetic value and pesticide residues in provisions collected by *Osmia bicornis* along a gradient of oilseed rape coverage

Anna Misiewicz[1]✉, Łukasz Mikołajczyk[1,2] & Agnieszka J. Bednarska[1]

Pollinators in agricultural landscapes are facing global decline and the main pressures include food scarcity and pesticide usage. Intensive agricultural landscapes may provide important food resources for wild pollinators via mass flowering crops. However, these are monofloral, short-term, and may contain pesticide residues. We explored how the landscape composition with a different proportion of oilseed rape (6–65%) around *Osmia bicornis* nests affects floral diversity, contamination with pesticides, and energetic value of provisions collected by this species of wild bees as food for their offspring. Altogether, the bees collected pollen from 28 plant taxa (6–15 per nest) and provisions were dominated by *Brassica napus* (6.0–54.2%, median 44.4%, 12 nests), *Quercus sp.* (1.2–19.4%, median 5.2%, 12 nests), *Ranunculus sp.* (0.4–42.7%, median 4.7%, 12 nests), Poaceae (1.2–59.9%, median 5.8%, 11 nests) and *Acer sp.* (0.6–42%, median 18.0%, 8 nests). Residues of 12 pesticides were found in provisions, with acetamiprid, azoxystrobin, boscalid, and dimethoate being the most frequently detected at concentrations up to 1.2, 198.4, 16.9 and 17.8 ng/g (median 0.3, 10.6, 11.3, 4.4 ng/g), respectively. Floral diversity and energetic value of provisions, but not the Pesticide Risk Index depended on landscape structure. Moreover, pollen diversity decreased, and energetic value increased with landscape diversity. Thus, even a structurally simple landscape may provide diverse food for *O. bicornis* if the nest is located close to a single but resource-diverse patch. Both *B. napus* and non-crop pollen were correlated with pesticide concentrations.

Pollinators provide essential ecosystem services for agricultural production[1], and a third of human food production benefits directly or indirectly from insect pollination[2]. However, in recent years, insects, including bee pollinators, have been exposed to many stressors and their biomass, abundance and species richness are declining over the world[3,4] with potentially detrimental effects on the ecosystem services they provide[5]. About 20% of pollination services in agricultural production are provided by wild bees[6]. A very important wild pollinator of various crops is the solitary bee *Osmia bicornis*[7–10], which is often a more effective pollinator than the honeybees[11].

A reduction in floral resource abundance and diversity observed in agroecosystems due to landscape simplification and habitat loss, together with widespread exposure to pesticides, are the main threats to pollinating insects[12]. Natural flower-rich habitats have been converted into large-scale agricultural monocultures in the last few decades[13]. Such large-scale crop monocultures are usually not attractive to pollinators due to the lack of floral resources (e.g., cereals) or, in case of mass flowering crops (e.g., oilseed rape), they provide short-lived, monofloral, and thus nutritionally unbalanced nectar and pollen resources[14–16]. Furthermore, mass-flowering crops are usually intensively treated with pesticides[12], which may increase pollinator mortality and could reduce their efficiency[17,18]. Pesticide residues were found both in the pollen of mass-flowering crops and in wild flowers growing in the field margins[19,20] and as many as 14 different compounds have been detected in winter *Brassica napus*[21]. *Brassica napus* is the second most essential oilseed crop and is considered the main valuable nectar-producing plant in the world[22]. However, the effect of the presence of oilseed rape around the nest on solitary bees is not fully clear. It was shown that proximity to oilseed rape crop positively affects the number of nesting

[1]Institute of Nature Conservation, Polish Academy of Sciences, A. Mickiewicza 33, 31-120 Kraków, Poland. [2]Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland. ✉email: misiewicz@iop.krakow.pl

O. bicornis, but it was suggested that oilseed rape benefits solitary bees in the form of abundant nectar for foraging flights rather than pollen for brood provisioning[23–25]. However, *B. napus* has been also identified as an important source of pollen for *O. bicornis* larvae[26–29]. The quality of pollen is very important to the larvae but, if contaminated with pesticides, it can affect negatively larval development[30]. Also the nutritional value of pollen may vary depending on the landscape[31].

The aim of this study was to investigate the influence of agricultural landscape structure with different proportion of oilseed rape crop in the area around *O. bicornis* nests on floral diversity, the level of contamination with pesticides and the energetic value of provisions retrieved from nests established in 12 sites. We hypothesized that an increasing proportion of oilseed rape around the nests reduces landscape heterogeneity and, in consequence, the pollen diversity of larval provision. Increased pesticide exposure risk was also expected, as the diets with higher proportion of oilseed rape pollen are more likely to be contaminated with pesticides. Because the diversity of floral resources may depend on the availability of different habitats around the nest, the effect of local landscape characteristic within the circular areas of 500 m and 1000 m around the nests, which correspond to the typical foraging distances of *O. bicornis*[26,32] was also studied. We hypothesized that landscape with lower proportion of oilseed rape crop in the area around *O. bicornis* nests and with more natural elements provides higher floral diversity, lower pesticide risk and better food quality in terms of its energy value.

Results

Floral diversity. Altogether, the bees collected pollen from 28 plant taxa (6–15 per nest), and three of them—*Brassica napus*, *Quercus sp*. and *Ranunculus sp*.—were recorded in all 12 nests (Table 1). Provisions were dominated by *B. napus* pollen, which constituted 6% to 54% (median 44.4%). Poaceae (1–60%, median 5.8%), *Ranunculus sp*. (0.4–43%, median 4.7%), *Acer sp*. (0.6–42%, median 18.0%), and *Quercus sp*. (1–19%, median

Pollen type	Mean proportion of plant taxa in provisions in different sites [%]												Min [%]	Max [%]	Median [%]
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12			
Brassicaceae/ *Brassica napus*	54.21	28.25	6.17	50.72	52.15	6.00	51.18	45.35	43.61	45.21	8.50	10.14	6.00	54.21	44.41
Acer sp.	30.74	–	28.30	18.56	17.38	–	42.31	4.74	–	4.71	0.59	–	0.59	42.31	17.79
Achillea typ	0.17	–	–	–	–	–	–	–	–	–	–	–	0.17	0.17	–
Aesculus sp.	0.17	0.41	–	–	0.19	–	0.54	–	–	–	0.79	–	0.17	0.79	0.41
Caprifoliaceae/ Lonicera	–	–	–	–	–	–	–	–	0.39	–	–	–	0.39	0.39	–
Carex sp.	–	–	–	–	–	–	–	0.38	–	7.90	–	3.11	0.38	7.90	3.11
Caryophyllaceae	–	0.41	0.17	0.41	–	–	–	–	–	–	–	–	0.17	0.41	0.41
Centaurea cyanus	–	–	–	–	–	–	–	0.19	–	–	–	–	0.19	0.19	–
Chenopodiaceae	–	–	0.17	–	–	–	–	–	–	–	–	–	0.17	0.17	–
Cornus sp.	–	–	–	–	–	–	–	0.38	–	–	–	–	0.38	0.38	–
Eleagnus sp.	–	–	–	–	–	–	–	–	–	0.34	–	–	0.39	0.34	–
Hypericum sp.	5.29	0.62	1.03	0.41	0.56	7.35	–	–	0.98	–	9.68	21.33	0.41	21.33	1.03
Juglans sp.	0.50	1.24	–	0.62	0.56	0.39	0.18	1.14	–	–	–	0.62	0.18	1.24	0.59
Lamium sp.	–	–	–	–	–	–	–	0.19	–	–	0.40	–	0.19	0.40	0.29
Malus sp.	–	–	–	–	–	–	–	–	0.79	0.50	–	–	0.50	0.79	–
Papaver sp.	–	0.82	0.17	–	–	0.58	–	0.38	0.20	–	–	–	0.17	0.82	0.38
Pinus sp.	–	–	0.17	0.21	0.19	–	–	0.19	–	0.17	0.40	–	0.17	0.40	0.19
Plantago sp.	0.50	1.24	–	–	–	0.39	–	–	–	0.34	0.59	1.45	0.34	1.45	0.54
Poaceae	1.16	8.87	1.20	3.71	5.79	25.73	–	17.46	2.36	5.21	59.88	27.74	1.20	59.88	5.79
Prunus sp.	0.83	2.27	–	0.21	0.75	1.55	–	2.28	0.59	2.69	3.36	3.73	0.21	3.73	1.91
Pyrus sp.	–	9.90	–	–	–	–	–	–	–	–	–	–	9.90	9.90	–
Quercus sp.	3.64	5.15	10.81	3.30	19.44	1.55	5.24	6.83	14.93	4.87	5.53	1.24	1.24	19.44	5.20
Ranunculus sp.	1.16	38.97	0.51	21.24	1.31	42.75	0.54	0.38	9.82	5.04	4.35	27.95	0.38	42.75	4.69
Rubus sp.	–	1.86	11.32	–	1.12	13.15	–	19.54	5.50	21.51	5.93	1.66	1.12	21.51	5.93
Rumex sp.	0.17	–	38.94	–	–	–	–	–	18.86	–	–	0.21	0.17	38.94	9.53
Salix sp.	1.49	–	0.17	–	0.19	–	–	–	0.39	0.17	–	0.62	0.17	1.49	0.29
Trifolium repens	–	–	0.86	0.62	0.37	0.58	–	0.57	1.57	0.67	–	0.21	0.21	1.57	0.60
Viola tricolor	–	–	–	–	–	–	–	–	–	0.67	–	–	0.67	0.67	–
PENS	3.54	5.51	4.98	3.88	4.00	4.82	2.53	4.94	5.32	5.68	4.32	5.92			
Provision energetic value [kJ/g]	18.62	18.02	17.74	18.32	18.77	17.58	17.15	18.18	18.19	17.46	18.08	17.41			

Table 1. Mean proportion of pollen types (identified to family, genus, or species level) in bee collected provisions per nest in the twelve nests (A1–A12) located in the agricultural landscape, pollen diversity expressed as pollen effective number of species (PENS), and provision energetic value.

5.2%) prevailed upon the rest pollen types, but up to 13 plant taxa contributed less than 1% to the diet of *O. bicornis* (Table 1). Pollen floral diversity (expressed as PENS) decreased with increasing LDI ($p = 0.011$) and FA1 ($p = 0.007$) in 500 m buffer (Fig. S2), although the significance of the latter relationship was driven by a single nest (A7 nest located in the site with high contribution of concrete, buildings, vegetation close to infrastructure and orchards; Fig. 1). The model including both explanatory variables i.e., LDI and FA1) was significant at $p = 0.0003$, $R^2 = 84\%$ (see Table S4 for β parameters). On a larger scale (1000 m buffer), PENS was not related to any of the four landscape variables (ORC, FA1, FA2, LDI).

Pesticide residues. Altogether, residues of 12 pesticides (eight fungicides, three insecticides, and one herbicide), 1–9 per nest, were detected in *O. bicornis* provisions at concentrations ranging from 0.11 ng/g (for acetamiprid) to 198.40 ng/g (for azoxystrobin). Acetamiprid was detected in 9 out of 12 nests and azoxystrobin, boscalid, and dimethoate were the next most frequently detected pesticides (7 out of 12 nests, Table 2).

No effect of any studied landscape variables (ORC, FA1, FA2, LDI) on the Pesticide Risk Index, either for 500 m or 1000 m buffer was found. Negative relationship of the Pesticide Risk Index with PENS (RMA regression, $p = 0.01$, Fig. S3) was found, however, although significant, the percent of explained variance was negligible

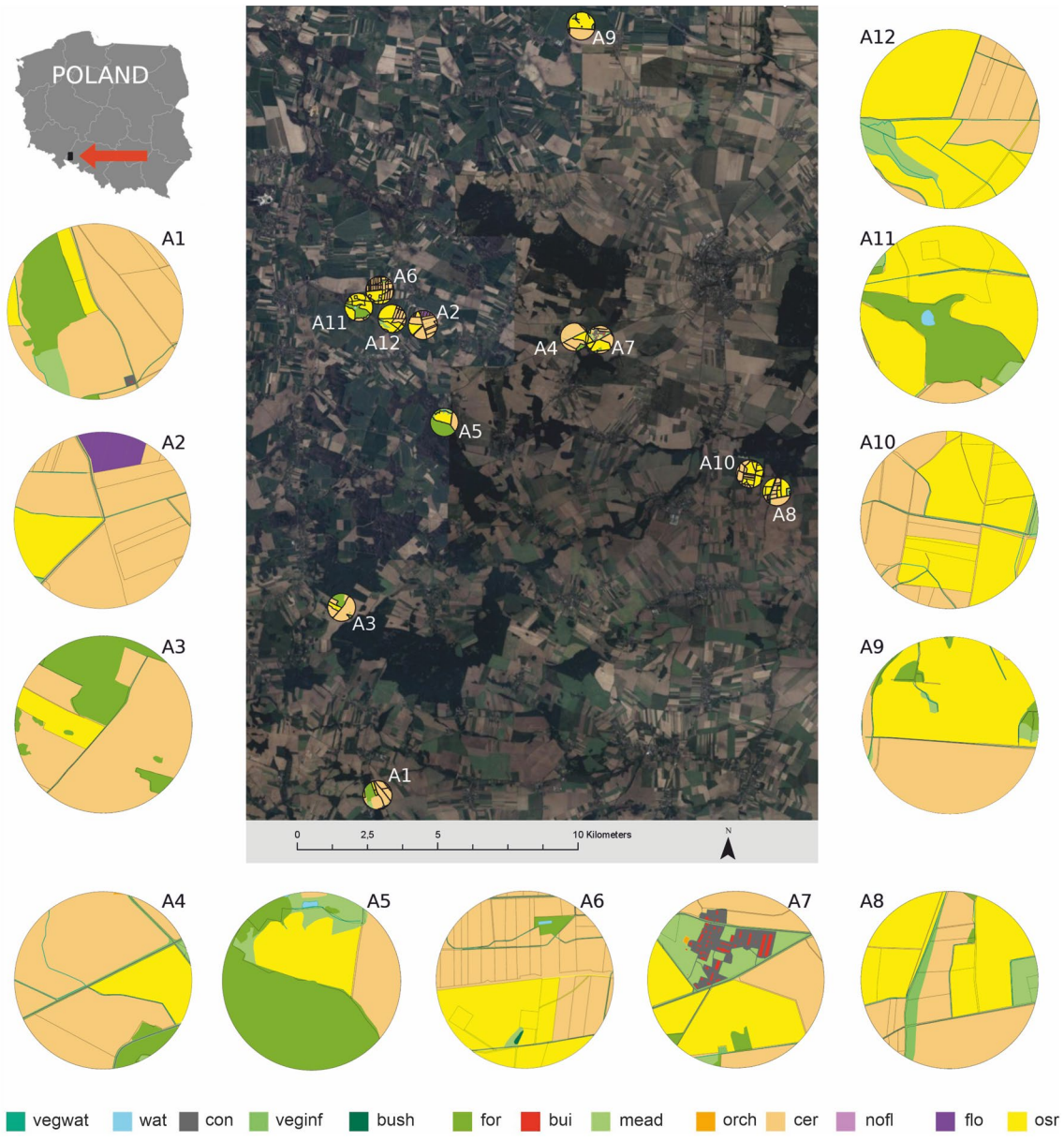

Figure 1. Location of the 12 study sites (A1–A12) in the agricultural landscape in the Opolskie province (Poland) and the characteristics of the 500 m buffer with the oilseed rape (yellow) and other 13 landscape elements (see Table S2 for detailed description). Map created with the use of Esri ArcMap 10 and GIMP 2.10.30 software. Satellite imagery data: Google, CNES / Airbus, Airbus Maxar Technologies obtained via Google Earth Pro software.

Active substance	Type[a]	Concentrations of active substances [ng/g] detected in provisions in different nests												LOQ [ng/g][b]	Number of nests detected	Percent of nests detected [%]	Min [ng/g][c]	Max [ng/g][c]	Median [ng/g]	Oral 48-h LD_{50} for honeybee [µg/bee][d]
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12							
Acetamiprid	I	–	0.37	–	0.83	–	0.28	0.33	0.12	0.11	1.16	0.11	0.22	0.10	9	75.00	0.11	1.16	0.28	14.53
Azoxystrobin	F	–	22.55	10.60	–	–	8.95	5.50	8.90	12.00	198.40	–	–	1.00	7	58.30	5.50	198.40	10.60	>25.00
Boscalid	F	15.45	10.20	15.75	–	16.95	11.25	–	–	–	–	7.90	5.90	0.01	7	58.30	5.90	16.95	11.25	100.00
Chlorothalonil	F	–	–	–	–	–	2.15	–	–	–	–	–	–	1.00	1	8.30	2.15	2.15	–	>40.00
Difenoconazole	F	–	–	–	–	–	–	6.55	–	–	40.30	–	–	0.01	2	16.70	6.55	40.30	23.43	>177.00
Fluopyram	F, N	–	4.93	–	41.10	–	2.88	12.23	–	–	–	10.03	2.08	1.00	6	50.00	2.08	41.10	7.48	>102.30
Fluxapyroxad	F	–	2.05	–	–	–	2.75	–	–	–	–	2.40	6.55	1.00	4	33.30	2.05	6.55	2.58	>110.90
Picoxystrobin	F	3.70	–	–	–	–	–	–	–	–	–	–	–	0.10	1	8.30	3.70	3.70	–	>200.00
Tebuconazole	F	24.35	–	16.20	–	–	–	10.45	18.55	–	6.30	–	–	1.00	5	41.70	6.30	24.35	16.20	>83.05
Dimethoate	I, A	–	2.75	–	12.05	–	7.85	4.35	17.08	–	–	1.70	3.00	1.00	7	58.30	1.70	17.08	4.35	0.10
Omethoate	I, A	–	1.13	–	6.33	–	4.98	1.40	9.13	–	–	–	1.33	5.00	6	50.00	1.13	9.13	3.19	0.05
Prosulfocarb	H	–	–	–	–	–	26.40	–	–	–	–	–	–	1.00	1	8.30	26.40	26.40	–	103.40
Provision mass [mg][e]		246.50	241.80	239.80	233.20	252.20	216.30	238.90	241.30	212.20	254.30	229.50	231.90							
Pesticide Risk Index[f]		0.01×10^{-3}	1.26×10^{-3}	0.02×10^{-3}	5.89×10^{-3}	0.004×10^{-3}	3.96×10^{-3}	1.75×10^{-3}	8.72×10^{-3}	0.01×10^{-3}	0.21×10^{-3}	0.40×10^{-3}	1.34×10^{-3}							

Table 2. Concentrations of active substances [ng/g] detected in provisions collected by *Osmia bicornis* bees as food for their larvae in twelve nests located in the agricultural landscape. Twelve active substances were detected above their limits of quantification (LOQ[b]), including 8 fungicides, 3 insecticides and 1 herbicide, and were used to calculate pesticide risk index with toxic unit (TU) approach based on 48-h LD_{50} values for orally exposed adult honeybee; '– ' means not detected. [a]I—Insecticide, H—Herbicide, F—Fungicide, N—Nematicide, A—Acaricide. [b]LOQ values for the 510 active substances tested can be found in Table S7 of the Supplementary materials in Bednarska et al.[26]. Four concentrations of omethoate were detected below the LOQ. [c]Min and max given for detected active substance. [d]Because neither larval nor adult LD_{50} values specific to *Osmia sp*. were available, the oral acute 48-h LD_{50} values for adult honeybees were used to calculate pesticide Risk Index. Oral acute 48-h LD_{50} values for adult honeybees available from the Pesticide Properties Database (https://sitem.herts.ac.uk/aeru/ppdb/en/Reports/321.htm). [e]Provision mass expressed as the average provision mass in the nest calculated based on data for the two types of cells: the most inner cells in the nesting cavities (i.e., those in which females would most likely develop) and the outermost cells located at the posterior end of the nesting cavity (i.e., those in which males would most likely develop) (*data not published*). [f]Pesticide Risk Index calculated for all detected active substances based on oral acute 48-h LD_{50} values for adult honeybee using Toxic Unit approach. To overcome the problem that in several cases the value for oral 48-h LD_{50} was expressed as > x, we used exactly the x value which is a conservative approach.

($R^2 = 0.2\%$). No relationship between the proportion of *B. napus* in pollen and Pesticide Risk Index (simple regression, $p = 0.6$) was found. RDA showed that the presence of both *B. napus* and non-crop pollen types are correlated with the concentrations of different pesticides (Fig. 2); the first ordination axis explained 29.1% of variance of the dependent variables and the second ordination axis explained 25% of variance. High correlation between plant taxa and pesticide was found especially for *Salix sp*. and picoxystorbin, Poaceae and fluxapyroxad, as well as *Carex sp*. and difenoconazole (Fig. 2).

Energetic value of provisions. The energetic value of provisions ranged from 17.15 kJ/g in the A7 nest to 18.77 kJ/g in the A5 nest (Table 1). A positive relationship was found between the energetic value of the provisions with the LDI ($p = 0.003$) and negative with FA1 ($p = 0.011$) (Fig. S4) for the 500 m buffer; the model including these two explanatory variables was significant at $p = 0.008$, $R^2 = 66\%$ (see Table S4 for β parameters). However, as in case of PENS, the relationship between energetic value of provisions and FA1 was mainly driven by A7 nest, which scored high on FA1 axis (Fig. S4). A highly significant negative relationship was observed between the energetic value and PENS (RMA regression, $p = 0.009$; Fig. S5), but, the model explained only 3% of the variability.

Discussion

The bees collected from 6 to 54% of *B. napus* pollen in the agricultural landscape with different proportion of oilseed rape around their nests. Teper and Biliński[29], who also studied *O. bicornis* pollen provisions during the flowering period of oilseed rape, found on average 46% of oilseed rape pollen and *Brassicaceae* was indicated as a one of the main sources of *O. bicornis* pollen by Haider et al.[27] and Peters et al.[28]. Albeit, even on sites dominated by oilseed rape (i.e., with ORC ≥ 40%), bees collected pollen from non-crop herbaceous plants (e.g., *Ranunculus sp*. at 43% in the A6 nest) and trees (mainly *Quercus sp*. and *Acer sp*. at 15% and 42% in the A7 nest, respectively), as mentioned by previous studies[33–36]. Studies by Coudrain et al.[23] conducted in agricultural areas with different percentage of forest around nests, showed a high proportion of *Ranunculus sp*. (58.6%) and *Quercus sp*. (23.4%) among 41 pollen types found in provisions of *O. bicornis*. In our study, *Quercus sp*. pollen was collected at relatively high proportion (1.2–19.4%) even by bees whose nests were adjacent to a field of oilseed rape. This

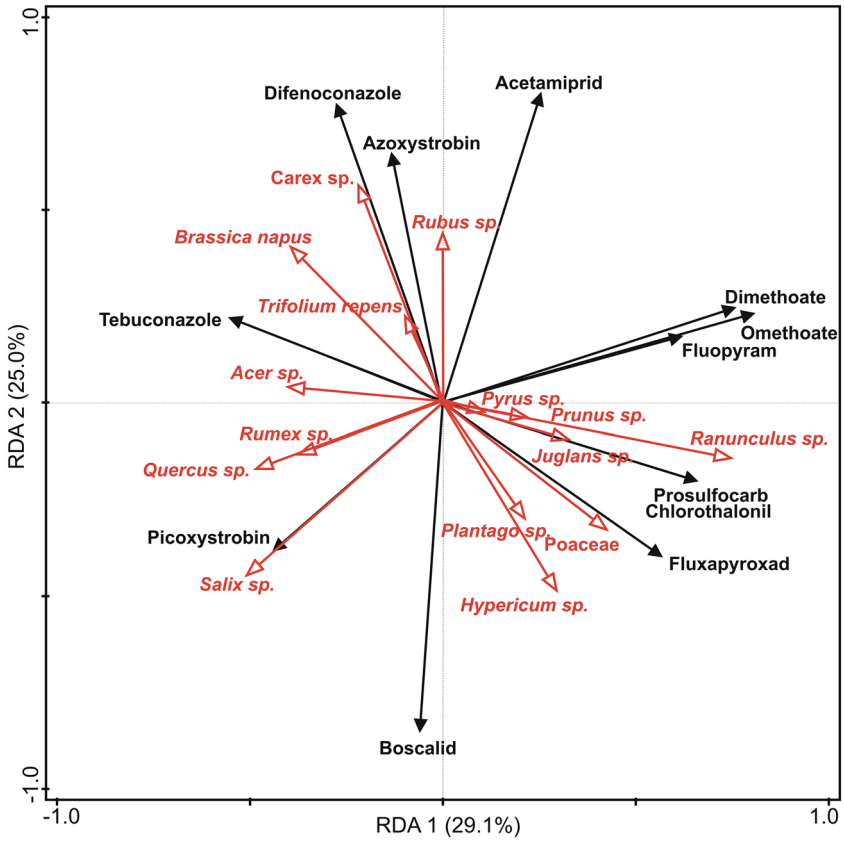

Figure 2. Results of a Redundancy Analysis (RDA) performed on the concentrations of pesticides in pollen, and the proprtion of pollen from the dominant taxa (i.e., more than 1% in at least one nest) in the studied 12 nests of *Osmia bicornis* in the agricultural landscape. Positions of the vectors of dependent variables on the two first RDA axes are shown by black arrows and that of the independent variables (proportion of pollen taxa) are shown by red arrows. The first ordination axis explained 29.1% of the variance of the dependent variables and the second 25.0%.

confirms previous observations that oak trees are a substantial source of pollen for *O. bicornis*[26,37,38] and that bees can fly large distances (up to 800 m from the nest, which is close to the maximum foraging distance of the red mason bee[32]), to reach oak pollen[26]. *O. bicornis* mixes different types of pollen to ensure constant protein content of provision[39], but Radmacher and Strom[36] suggested that because wind-pollinated oak trees offer large amounts of pollen, *O. bicornis* females may temporarily (and locally) specialize in one or two plant species with high pollen availability to maximize the collected pollen mass per unit time.

Saunders et al.[40] found that bees visit ca. 100 wind-pollinated plant genera, and large part of visitation records were for grasses and sedges (Poales). Schulze-Albuquerque et al.[41] indicated that the floral cues, colour, and scent of Poaceae can attract insects. For example, honeybees, bumblebees and sweat bees (*Lasioglossum spp*.) foraged on a pasture grass from Poaceae family[42] and Poaceae was one of the dominant pollen type (4–12%) in the diet of the Australian bee *Tetragonula carbonaria*[43]. In our study, Poaceae pollen was found in 11 out of 12 nests and accounted for up to 60% of pollen provisions (Table 1). The presence of Poaceae, but at smaller proportions (0.3–4.7%), in the *O. bicornis* diet was also confirmed by Splitt et al.[44].

Floral diversity of pollen provisions decreased on sites with a greater share of "urban landscape" features (i.e., buildings, concrete and infrastructure, vegetation by infrastructure, orchards) and increased on sites with a higher share of vegetation close to water bodies and borders between fields and natural habitats. The significance of this relationship was driven mostly by a single nest located close to build-up area with a high share of buildings, concreate, and in-between vegetation as well as orchards. This result shows the imporatnce of more natural landscape elements for the floral diversity of wild bee collcetd pollen, however, a previous study perfomerd in different agricultural landscpaes that used similar landscape elements, showed an increase in pollen diversity with higher proportion of built-up areas around the nest[26]. Moreover, while in Factor Analysis all 14 landscape elements were included, the LDI included only those 7 landscape characteristics that are expected to be functionally relevant for the red mason bee (Table S1) and still a negative relationship with PENS was found. This may indicate that even a homogeneous landscape always contains some portion of semi-natural habitats that provide food diversity, which was also suggested by Malagnini et al.[45] in their study of diversity of pollen collected by honeybees in an agricultural area. Malagnini et al.[45] expressed the landscape heterogeneity around honeybee nests by both landscape composition through Principal Component Analysis and landscape diversity through Shannon diversity index (based on data for 24 land-use classes (elements). Comparably to our study, the authors expected to find highly diverse landscapes offering a wider range of pollen types in comparison to homogeneous landscapes. However, honeybees collected highly diverse pollen regardless of the landscape diversity, while landscape composition affected pollen diversity only at the end of the flowering season when the proportion of semi-natural areas started to play important role[45]. Also, the study by Danner et al.[46] on honeybees found that pollen composition was not affected by landscape composition expressed via the Shannon diversity index. These results question the validity of using landscape diversity indices calculated based on the type of the element and its coverage to describe landscape diversity available for bees artificially introduced into the environment[45,47]. Unlike local populations, bees artificially introduced to the field together with nesting material for one season, are not constrained by nest availability, and their reproductive success mainly depends only on the degree to which a landscape facilitates or impedes access to the resource patch(es) and/or movement of bees among resource patches (nectar and pollen) (i.e., connectivity). In this case, even the existence of a single element (patch) in a small proportion (e.g., only a small multi-species flowering meadow which may contain an average 60 plant species[48]) can provide a more diverse food source than several elements (patches), which provide little diverse food (e.g., single-species strip of trees or shrubbery, monoculture of flowering crop, single-species orchard, etc.). On the other hand, LDI based on Shannon diversity index will not capture the diversity of the multi-species flower meadow, as it will treat it as a single element (patch) functionally relevant for bees. Because LDI calculated for sites dominated by a single element (patch) will be lower than for sites with several bee-relevant elements, it may produce results opposite to the expected increase in pollen diversity (PENS) with landscape diversity (LDI). Shannon diversity index quantifies the heterogeneity of landscapes, considering both richness and evenness of land-use elements (patch types), with low values of the index indicating a low landscape heterogeneity, but it does not consider species richness of the individual elements themselves. Therefore, although the widely used Shannon index has been recommended for landscape management within an ecological framework, description and interpretation of the relationships between pollen diversity (but also other variables) and Shannon-based landscape diversity indices[49], should be made with caution. On a larger scale (1000 m buffer), our results show that floral diversity of pollen was not related to any of the four landscape variables studied. This emphasizes the importance of the local landscape, (i.e., the area in the close vicinity of the nest) for the food resources of *O. bicornis*.

Bees may be frequently exposed to different classes of pesticides through nectar, pollen, and guttation droplets[21,50–52]. We found residues of 12 pesticides in *O. bicornis* provisions with acetamiprid being the most frequently detected and dimethoate and omethoate presenting the highest risk to bees (i.e., their contribution to the pesticide risk index was the largest). The reported concentrations of acetamiprid residues detected in pollen directly collected from plants were in the range 0.02–0.82 ng/g[19,53], similar to what we found in provisions collected by *O. bicornis* in this study (up to 0.83 ng/g, median 0.28 ng/g) and in the earlier study by Bednarska et al.[26]: 0.1–2.23 ng/g (median 0.30 ng/g). Acetamiprid belongs to neonicotinoids, which are the most widely used insecticides in the world[54]. It was proven that acetamiprid has a negative effect on adult honeybees and stingless bees, including a significantly reduced lifespan and affected locomotor activity[55,56]. In case of *O. bicornis*, Mokkapati et al.[30] showed that although acetamiprid did not affect larval survival and larval body mass, the length of larval stage (i.e., time to cocoon formation) was significantly shorter in larvae exposed to acetamiprid compared to controls. The negative effect of other pesticides detected in our pollen samples, such as picoxystrobin and dimethoate was also confirmed in studies on adult honeybees fed ad libitum sucrose solutions containing different concentrations of these insecticides[57–59].

Our results showed that bees are exposed to a wide spectrum of pesticides in agricultural landscapes, as previously indicated in honeybee studies[50,60]. In contrast to honeybee pollen, the one collected by solitary bees in the agricultural landscape was less frequently evaluated for pesticide residues. Bednarska et al.[26] detected residues of 34 pesticides (with acetamiprid among the 10 found most often) in provisions collected by *O. bicornis* over the entire season in the intensively used agricultural landscape in Poland. Also, Rundlöf et al.[61] found residues of 12 pesticides in provisions collected by *O. lignaria*, which experienced similar pesticide risk at sites without and with flower strips used to mitigate the effects of bee pesticide exposure and support bee reproduction in intensively farmed landscapes in Sweden. Centrella et al.[62] found 28 pesticides (13 insecticides and 15 fungicides) in pollen collected by *O. cornifrons* in apple orchards and indicated that the presence of agricultural habitats within 2 km was associated with an increased level of pesticide risk. In our study, there was no effect of any of the evaluated landscape variables on the Pesticide Risk Index, including both 500 m and 1000 m buffer.

Mass-flowering crops, such as oilseed rape, are often intensively treated with pesticides[21,63]. Zioga et al.[21] indicated up to 14 different compounds in winter *B. napus*, and the median concentrations of these compounds found in cultivated plants was higher than those in wild plants. Similarly, in individual oilseed rape pollen samples collected in China, Wen et al.[63] found residues of at least 10 pesticides, and 4 samples contained up to 40 pesticides. It is not clear whether the pesticide residues found in our study came from contaminated oilseed rape flowers, other non-focal crops, wildflowers along field margins, or other sources, since the pesticide analysis could be performed only on mixed pollen from multiple provisions from each nest. Although no relationship between the proportion of *B. napus* in pollen and Pesticide Risk Index was found, we cannot exclude that even a small amounts of crop pollen collected by bees can lead to significant pesticide risks[15]. Moreover, we observed negative relationship between PENS and Pesticide Risk Index, showing that reduced pollen diversity (PENS) increases pesticide risk in bee collected pollen. However, this relationship explained only a small percentage of the total variance and seems to be driven by high contribution of dimethoate and omethoate to the Pesticide Risk Index. The presence of these two active substances cannot be directly linked to any specific pollen species and, in fact, the RDA analysis showed that the presence of both *B. napus* and non-crop pollen types are correlated with the concentrations of different pesticides. Contrary to our results, a positive relationship between pollen diversity and insecticide risk levels in *O. bicornis* pollen was found by Bednarska et al.[26] which may also suggest the contamination of plants in non-crop areas.

The energetic values of provisions were similar to those estimated for honeybee pollen (16.6–17.1 kJ/g) collected by beekeepers in Portugal[64]. The energetic value of pollen increased with increasing landscpe diversity and on sites with a higher share of vegetation close to water bodies and borders between fields and natural habitats and decreased on sites with a higher share of "urban landscape" features, namely was the lowest on A7 site. Surprisingly, the provision of nest A7, although located close to "build-up areas", was dominated by theree pollen types (*B. napus* > *Acer sp.* > *Quercus sp*), was the least diverse (had the lowest value of PENS) and had the lowest caloric value.

Despite the low percentage of explained variance, the negative relationship between PENS and energetic value may show that a more diverse pollen provision does not necessarily show better quality in terms of caloric value. It should be noted, that bomb calorimetry does not necessarily correspond to digestible energy, as it measures total energetic value of a sample, including also poorly digestible parts of the pollen grain, such as the pollen wall[65]. Furthermore, the presence and number of pores of germination has been hypothesized to influence pollen digestibility[65]. At the same time, provisions taken from *O. bicornis* brood cells may contain nectar sugars, which also contribute to the caloric value of provisions, but no study has specifically determined the ratios of pollen to nectar in *O. bicornis* provisions and the factors that control that ratio. Maddocks and Paulus[66] suggested that *O. bicornis* provision brood cells with pollen and a comparatively low proportion of nectar (2%), but in a study performed on the larvae of the alfalfa leaf-cutting bee, *Megachile rotundata* (which belongs to the same Megachilidae family as *O. bicornis*), Cane et al.[67] estimated that the provisions consist of pollen and nectar at a 1:2 ratio (~ 33% alfalafa pollen and 67% nectar). *Brassica napus* pollen has a high energy value (12.6 kJ/g) and a high fat content (5.47%)[68], so even a small proportion of oilseed rape pollen might influence mean calorific value of larval provision. Nevertheless, it is unlikely that proportion of *B. napus* determines the energetic value of the nest provisions in our study, as a proportion of *B. napus* in the nest with the lowest caloric value of the provision (51% in A7 nest) was similar to that of nests with the highest caloric values (52%, 54% and 50% in nests A5, A1 and A4, respectively).

Conclusions

In conclusion, although *O. bicornis* is a generalist species, we confirmed that it prefers a certain set of plants, including trees and shrubs, if available. The pollen collected by *O. bicornis* was dominated by five taxa, including *B. napus*, by the fields of which, nests were placed during its flowering period. Both floral diversity and energy value of provisions were related to the landscape structure. The influence of the landscape structure and diversity was visible on a small scale (500 m buffer) only, which is in line with the rather small foraging radius of that species[32,69]. However, caution is needed for the interpretation of the results based on relationships with the Shannon-based landscape diversity index, as it does not consider species diversity within individual landscape elements (cover types). In our study, the presence of landscape elements, their sizes (i.e., proportions in the landscape) and connectivity between different landscape elements were captured by the scores for both FA1 and F2, but the results for relationship of PENS with LDI indicate that it is still necessary to include the quality (e.g., diversity) of landscape elements themselves. Although time-consuming, measures based on provisions collected by bees for their offspring rather than landscape characteristics provide a complete picture of food resources in an agricultural landscape. After all, it is what the bees have collected, regardless of where they collected it, that determines the survival and development of the offspring in the nest. We showed that bee larvae

are exposed through their food to a variety of pesticides, the concentrations of which are correlated with both crop pollen (*B. napus*) and other non-crop plants (e.g., *Ranunculs sp*., Poaceae, *Carex sp*.). Although both the mass-flowering crops and the nearby flowers and trees can be contaminated with a wide range of pesticides, in the studied landscape the pesticide risk generally decreased with increasing floral diversity of provisions. Thus, introduction of varied flora into the agricultural landscape should be considered in pollinator conservation and management decisions to mitigate the effects of agricultural landscape.

Methods

Sites and landscape characteristic. Data were collected during oilseed rape blooming season in 2019 from twelve sites located in the agricultural landscape of the Opolskie province, Poland (Fig. 1). The sites represented the gradient of oilseed rape coverage (ORC, 6–65%) within non-overlapping circular areas of 500 m radius (called the "buffer" thereafter) (Table S1). The local landscape structure around each nest was characterised based on land cover maps created at two spatial scales (500 m and 1000 m buffers), using 13 discrete, non-overlapping landscape elements (land cover types) and two linear features representing land fragmentation (Table S2). It was analysed in ArcMap 10[70] as described in Misiewicz et al.[71]. Landscape elements (without oilseed rape coverage which was used separately due to its importance for bees and as a controlled experimental factor) were reduced to two factors (FA1 and FA2) using Factor Analysis, which explained respectively 32.4% and 21.0% of the total variability in local landscape characteristics in the 500 m buffer, and 29.0% and 27.1% respectively in the 1000 m buffer. FA1 for the 1000 m buffer and FA2 for the 500 m buffer captured almost the same landscape elements, which scored similarly: "arable lands" features (i.e., cereals, nonflowering and flowering crops but also bushes and the length of borders between fields) scored high, while "landscape naturalness" (meadows, forests, and the length of borders between fields and natural habitats) scored low on those axes. However, FA2 for 1000 m buffer and FA1 for 500 m buffer were inversed: in general, landscape elements characteristic for "urban areas" (concreate, buildings, but also vegetation close to infrastructure) that scored high on FA1 for 500 m buffer, scored low on FA2 for 1000 m and, at the same time, those scored low on FA1 for 500 m (water and vegetation by water) scored high for FA2 for 1000 m. Only some elements (e.g., orchards) shifted their position in FA1 and FA2 factors, scoring higher either in "built-up areas" (FA1, buffer 500 m) or "arable lands" (FA1, buffer 1000 m). See Misiewicz et al.[71] for more details on Factor Analysis. In addition, the Landscape Diversity Index (LDI, i.e., Shannon-Wiener index) of seven landscape elements that present potential foraging habitats for bees (i.e., vegetation by water, vegetation by infrastructure, bushes, forests, meadows, orchards, flowering crops; Table S2) was calculated for each study site.

Solitary bees and experimental design. One artificial nest (Fig. S1A) with 16 nesting cases providing 360 nest cavities, and ca. 550 commercially available cocoons of *O. bicornis* (Pszczelinka, Kapka Sp. z. o.o., Poland) were placed on the perimeters of the oilseed rape field in each site centre. The nests were left in the field from 17th April to 4th June 2019. In agricultural landscapes, flower resources and pesticide use change over space and time[72]. Thus, flower phenology influences bee activity and expected pesticide exposure[73]. To ensure the availability of food resources in the close vicinity from the nest, we allowed females to gather food for their larvae only during the restricted period of oilseed rape blooming.

Upon transferring to the laboratory, half of each nest (8 upper nesting cases) was kept under changing temperature conditions to breed the next generation of bees as described by Misiewicz et al.[71] and the second half (8 lower nesting cases) was frozen at – 20°C for pollen provision sampling (Fig. S1B). The samples of provisions were kept in the freezer for 4 months before used for chemical analysis.

Eggs or larvae were removed from the brood cells and the pollen provision from a separate nesting cavity was placed in a separate Eppendorf tube and stored at – 20°C until further analysis. For this study, only nesting cavities with less than six provisions per cavity were used (27–104 cavities per nest; Table S3); the remaining cavities were used for another study. The pollen provisions were thoroughly mixed to create a combined representative sample for the entire nest. Each combined sample was divided into three subsamples used for palynological analysis (~ 3 g), pesticide analysis (~ 30 g) and to determine pollen energetic values (~ 0.4 g).

Palynological analysis. The palynological analysis was performed using microscope slides, following the method described in the Supplementary Materials. *Brassica napus*, *Centaurea cyanus*, *Trifolium repens,* and *Viola tricolor* were identified at the species level and other taxa at the genus (20) or family level (4). All pollen types for each nest site are presented in Table 1. The pollen effective number of species (PENS)[26] was calculated for each nest as exp(H'), where H' is the Shannon-Wiener diversity index[74,75].

Pesticide analysis. For pesticide analysis, pollen samples were screened for residues of 510 different active substances using LC–MS/MS or GC–MS/MS techniques at the Institute of Plant Protection, National Research Institute, Laboratory of Food and Feed Safety, Białystok, Poland (see Bednarska et al.[26] for all details on chemical analysis, including multiresidues and single methods used, LC–MS/MS and GC–MS/MS parameters and validation parameters for 510 active substances analyzed (LOQ levels and recovery (%)). The results were reported as the mean value of two parallel determinations for each nest and a site Pesticide Risk Index was calculated using toxic unit (TU) approach as described by Bednarska et al.[26] to capture the combined hazard and exposure level to multiple substances at a site. In short, the TU for each nest was calculated as the sum of the products of the concentration of each active substance and the mean provision mass per larvae divided by the oral LD_{50} of that active substance for adult honeybees (Table 2), using a following equation:

$$\text{Pesticide Risk Index} = \sum \frac{\text{Acive substance}\left[\frac{ng}{g}\right] \times \text{provision mass}[g]}{\text{LD}_{50}\,\frac{ng}{\text{bee}}}$$

The mean provision mass still available for larvae in each nest was calculated from the provision mass collected from those nesting cavities which contained six or more brood cells and were used for another study (see Table 2).

Determination of pollen energetic values. The energetic value of the vacuum-dried provision samples was measured with a Semimicro Calorimeter (model 6725) containing a calorimeter thermometer (model 6772) and a Semimicro Oxygen Bomb (model 1109A) (Parr Instrument Company). The energetic value of the pollen in each nest was measured in 3 replicates and expressed in kJ/g dry mass (Table 1).

Statistical analysis. For each response variable (PENS, Pesticide Risk Index, energetic value), multiple regression analyses with all landscape variables (i.e., ORC, FA1, FA2, and LDI) as explanatory variables were performed separately for 500 m and 1000 m buffers. Landscape variables were standardised, and the stepwise backward selection process was used to remove nonsignificant variables from the model so that only variables significant at $p \leq 0.05$ remained. The normal distribution of residuals was tested for each model using the Shapiro–Wilk test.

The relationship between pollen diversity (PENS) and Pesticide Risk Index (TU) was analysed using reduced major axis (RMA) regression to test whether reduced pollen diversity increases pesticide risk in bee-collected pollen. The RMA was also used to test relationship between PENS and the energetic value of provision. The RMA was used instead of standard least-squares regression to handle errors in both the x and y variables.

Because we hypothesized that prevalence of oilseed rape pollen in the provisions would increase pesticide risk due to pesticide applications on oilseed rape fields, we tested whether the Pesticide Risk Index depends on the proportion of *B. napus* pollen found in the provisions by using a simple regression analysis. Moreover, a redundancy analysis (RDA) with Monte Carlo test with 499 unrestricted permutations was performed to determine the pattern of variability in pesticides concentrations among sites by the proportion of plant taxa as explanatory variables. For RDA we selected pollen contributing more than 5% to the diets in any nest (i.e., 13 pollen types were not included in this analysis).

Multiple regression analyses and simple regression analyses were performed using Statgraphics Centurion 18 (StatPoint, Herndon, VA, USA; http://www.statgraphics.com), RMA regression was performed using PAST 3 software for Windows (https://softfamous.com/past/) and RDA analysis was performed in Canoco ver. 5[76].

Data availability

The raw data are available from the corresponding author upon request.

Received: 17 May 2023; Accepted: 2 August 2023
Published online: 17 August 2023

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Acknowledgements

We would like to thank Ryszard Laskowski from the Institute of Environmental Sciences, Jagiellonian University, for constructive comments. This study was supported by the National Science Centre, Poland within SONATA 13 (2017/26/D/NZ8/00606) and by the subsidy for scientific activity of the Jagiellonian University (N18/DBS/000003).

Author contributions

A.J.B.: Conceptualization, Writing—review & editing, Supervision, Funding acquisition. A.M.: Methodology, Writing—original draft. A.J.B. and A.M.: Formal analysis. Ł.M. and A.M.: Investigation, Visualization, Data curation.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-023-39950-5.

Correspondence and requests for materials should be addressed to A.M.

SUPPLEMENTARY MATERIALS

Floral resources, energetic value and pesticide residues in provisions collected by *Osmia bicornis* along a gradient of oilseed rape coverage

Anna Misiewicz[1*], Łukasz Mikołajczyk[1,2], Agnieszka J. Bednarska[1]

[1]Institute of Nature Conservation, Polish Academy of Sciences, A. Mickiewicza 33, 31-120 Kraków, Poland

[2]Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

[*]misiewicz@iop.krakow.pl

Methods

1. Palynological analysis

For palynological analysis, 3 g of composite pollen sample from each pooled collection was taken and mixed with 100 ml of distilled water and vortexed for several times, each time for 2 minutes, over the course of 6 hours. The resulting solution was used to prepare microscope slides[1], two slides per sample. To determine the share of pollen representing different taxa, ca. 300 grains per slide were counted[2] along two lines chosen randomly across the cover slip at a magnification of 400x (Olympus BX41) using reference specimens and published reference collections. *Brassica napus* (oilseed rape), *Centaurea cyanus*, *Trifolium repens*, and *Viola tricolor* were identified at the species level and other taxa to the genus or family level. Average sum of pollen grains from two analyzes was calculated for each taxon and the data were expressed as the percentage content of individual type of pollen.

2. Pesticide analysis

The pesticide analyses were performed using the protocols and methodology fully described in Supplementary materials in **Bednarska *et al.***[3].

Tables

Table S1. Characteristics of the study sites in the close vicinity of *Osmia bicornis* nests (i.e., within 500 m and 1000 m radius around each nest, called "buffers") in the agricultural landscape selected for the field study in 2019; ORC – oilseed rape coverage [%], LDI – Landscape Diversity Index.

Nest ID	Agricultural area [%] within 5x5 km around the nest	Natural area [%] within 5x5 km around the nest	ORC [%] in 500 m buffer	ORC [%] in 1000 m buffer	LDI in 500 m buffer	LDI in 1000 m buffer
A1	88	10	6.35	6.73	3.22	3.20
A2	91	12	13.93	11.83	2.24	3.07
A3	57	44	14.33	8.20	1.94	1.86
A4	81	21	14.98	22.93	2.53	3.14
A5	71	31	22.48	10.77	2.55	2.80
A6	93	11	39.56	42.87	1.69	2.09
A7	84	17	39.98	20.26	2.53	3.24
A8	80	21	45.73	22.62	1.66	3.08
A9	94	6	52.85	28.16	1.56	2.09
A10	81	20	57.33	30.15	1.21	2.94
A11	91	15	63.23	35.27	2.13	2.79
A12	92	12	65.30	39.02	1.52	2.47

Table S2. Description of landscape characteristics (elements) used for characterization of buffers around each *Osmia bicornis* nest as described in Mikołajczyk *et al*.[4]. The landscape elements (cover types) used to calculate Landscape Diversity Index (LDI) are in boldface.

Landscape unit	Name	Acronym	Description
1	**Vegetation by water bodies**	**vegwat**	**Encompasses (an arbitrarily chosen if not already mapped) 2 m wide strip of terrain that surrounds lakes, ponds, and runs along both sides o streams, brooks, rivers, drainage ditches and hydro-technical channels of a different sort. Because of its peripheral location, this type of vegetation is rarely maintained or cut and seems to remain in a relatively untouched state throughout the whole year.**
2	Water bodies	wat	Groups all bodies of water, flowing and standing, of natural and anthropogenic origin.
3	Concrete, asphalt, infrastructure	con	Groups all anthropogenically paved terrains – roads, walkways, paved yards, and other infrastructural objects like pylons, wind turbines, and transmission towers.
4	**Vegetation by infrastructure**	**veginf**	**Groups vegetation around roads, walkways, yards, and infrastructural objects. This vegetation is often maintained is some way (roads) but might as well stay forsaken (back yards).**
5	**Bushes**	**bush**	**Groups terrains covered with perennial plants, bushes, shrubs, overgrown uncultivated lands but not yet forests. Group gathers also urban parks and cemeteries.**
6	**Forests**	**for**	**Groups terrains covered with trees and underbrush.**
7	Buildings	bui	Gathers man-made structures of habitual or industrial character – edifices, houses, factories, warehouses, etc.
8	**Meadows**	**mea**	**Groups terrains covered by grasslands and meadows, offering an abundance of flowering plants when not maintained.**
9	**Orchards**	**orch**	**Groups terrains with perennial, flowering fruits plantations.**
10	Cereals	cer	Gathers agricultural land with anemophilous grasses cultivated for grain. Terrains prone to agricultural treatment (e.g., insecticide spraying).
11	Non-flowering crops	noflo	Groups agricultural land with crops not producing regular flowers or harvested before blooming. Terrains prone to agricultural treatment.
12	**Flowering crops**	**flo**	**Gathers agricultural land with crops producing flowers. Terrains prone to agricultural treatment.**

13	Oilseed rape	oil	Groups agricultural land planted with intensively flowering oilseed rape (*Brassica napus*). Prone to heavy agricultural treatment.
14	Field-to-field borders	ff	Counts the total length of field boundaries and is used as a proxy for average agricultural plot size and land fragmentation.
15	Field-to-natural borders	fn	Counts the total length of boundaries between agricultural land and natural (or semi-natural) habitats and is used as a proxy for potential shelter availability for arthropods in an agricultural landscape.

Table S3. Number of nesting cavities per nest of *Osmia bicornis* used to prepare mixed provision samples for palynological analysis, screening of active substances and energetic value measurements.

Nest ID	No. of total nesting cavities with provisions	No. of nesting cavities with provisions selected for analysis in this study
A1	124	104
A2	76	40
A3	100	60
A4	74	38
A5	75	64
A6	106	68
A7	60	30
A8	104	62
A9	47	27
A10	60	38
A11	147	73
A12	138	64
Sum	**1111**	**668**

Table S4. Results of the backward stepwise multiple regression analysis for 500 m buffer to describe the relationship between explanatory variables, i.e., Pollen Effective Number of Species (PENS), Pesticide Risk Index and energetic value of pollen and four independent variables (oilseed rape coverage (ORC, %), FA1, FA2 and Landscape Diversity Index (LDI)). The regression parameters b and β (the latter for the model on standardized variables) and p values are reported only for the variables included in the final model containing only significant explanatory variables (at $p \leq 0.05$); p, R^2, R^2_{adj} – values for the final model. *NS* – not significant.

Explanatory variable		Independent variable						
		ORC	FA1	FA2	LDI	p	R^2	R^2_{adj}
PENS	p	–	0.007	–	0.011			
	b	–	-0.121	–	-0.867	0.0003	83.8%	80.2%
	β	–	-0.548	–	-0.499			
Pesticide Risk Index	p	–	–	–	–	*NS*	–	–
	b							
	β							
Energetic value	p	–	0.011	–	0.003			
	b	–	781.370	–	-79.790	0.0078	66.0%	58.4%
	β	–	-361.730	–	449.480			

Figures

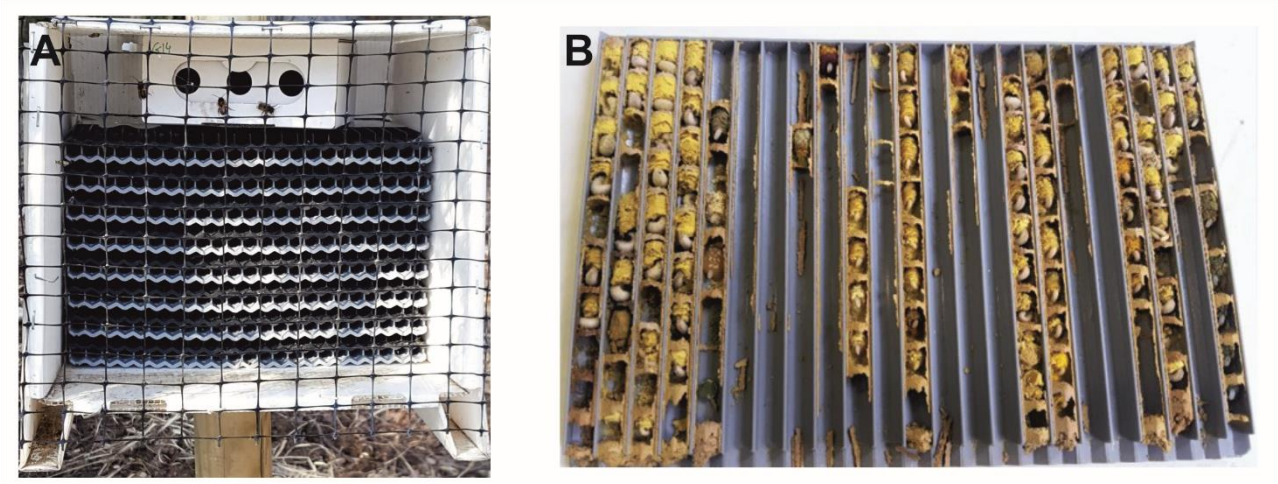

Figure S1. (A) The artificial nest made of 16 polystyrene elements (nesting cases), stacked on top of each other. Nesting cases were placed in the box-shaped housing made of durable and weather-resistant polypropylene together with a carton box with *Osmia bicornis* cocooned adults. To protect solitary bees against birds or rodents, each nest was closed with a plastic grid (1×1cm) and attached to a wooden pole at a height of ca. 1 m above the ground. (B) Example of a nesting case with pollen provisions stored.

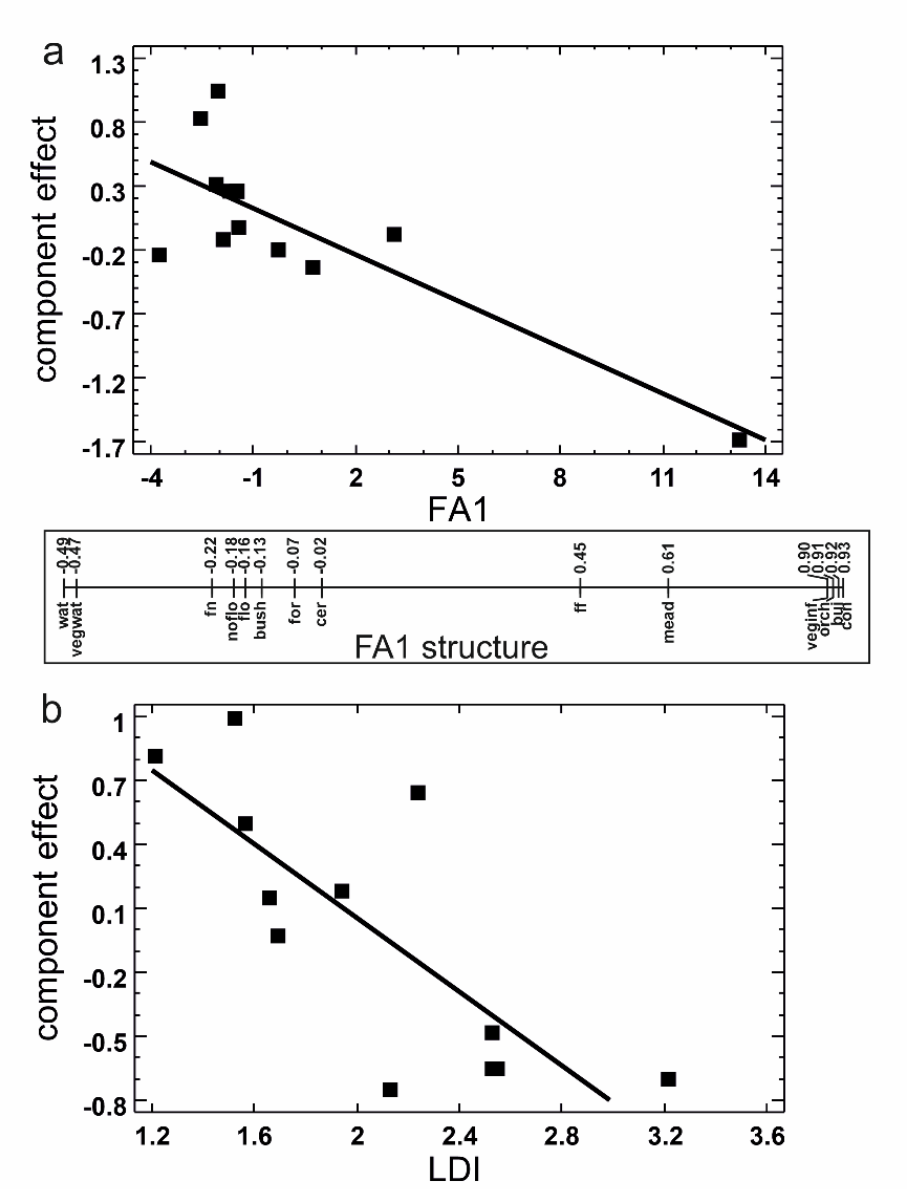

Figure S2. Results of the multiple regression analysis for the 500 m buffer: negative effects of (A) FA1 ($p = 0.007$) and (B) LDI ($p = 0.011$) on the pollen diversity (PENS). The overall model including both variables was significant at $p \leq 0.001$ and explained 83.8% of the variability. The line shows the relative change in the predicted values of the PENS when changing (A) FA1 or (B) LDI over their observed ranges. Each point (site) is then plotted by adding its residuals to a line. Note that the values on the y-axis are the residuals of the part of the model explained by another significant variable. The right side of the graph A shows the variables scores for 14 landscape elements that describe sites (*see Table S2 for a full description of the landscape units*) spread on the unitless FA1 axis.

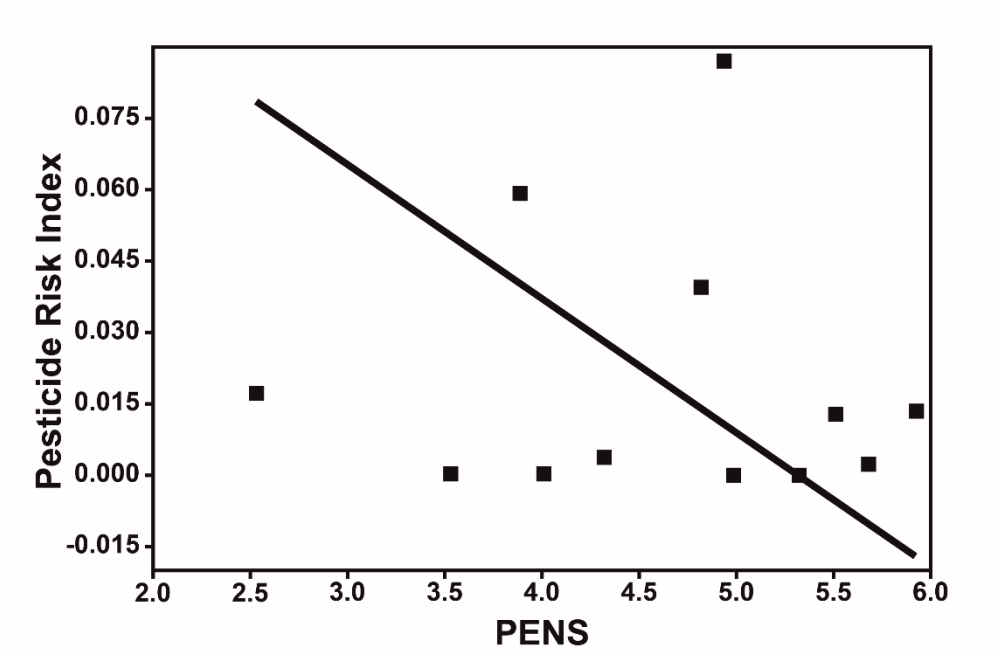

Figure S3. Results of the reduced major axis (RMA) regression: negative relationship between pollen diversity expressed as pollen effective number of species (PENS) and Pesticide Risk Index expressed as toxic unit ($p = 0.01$) analysed in pollen provisions collected by *Osmia bicornis* for their larvae in 12 nests located in agricultural landscape.

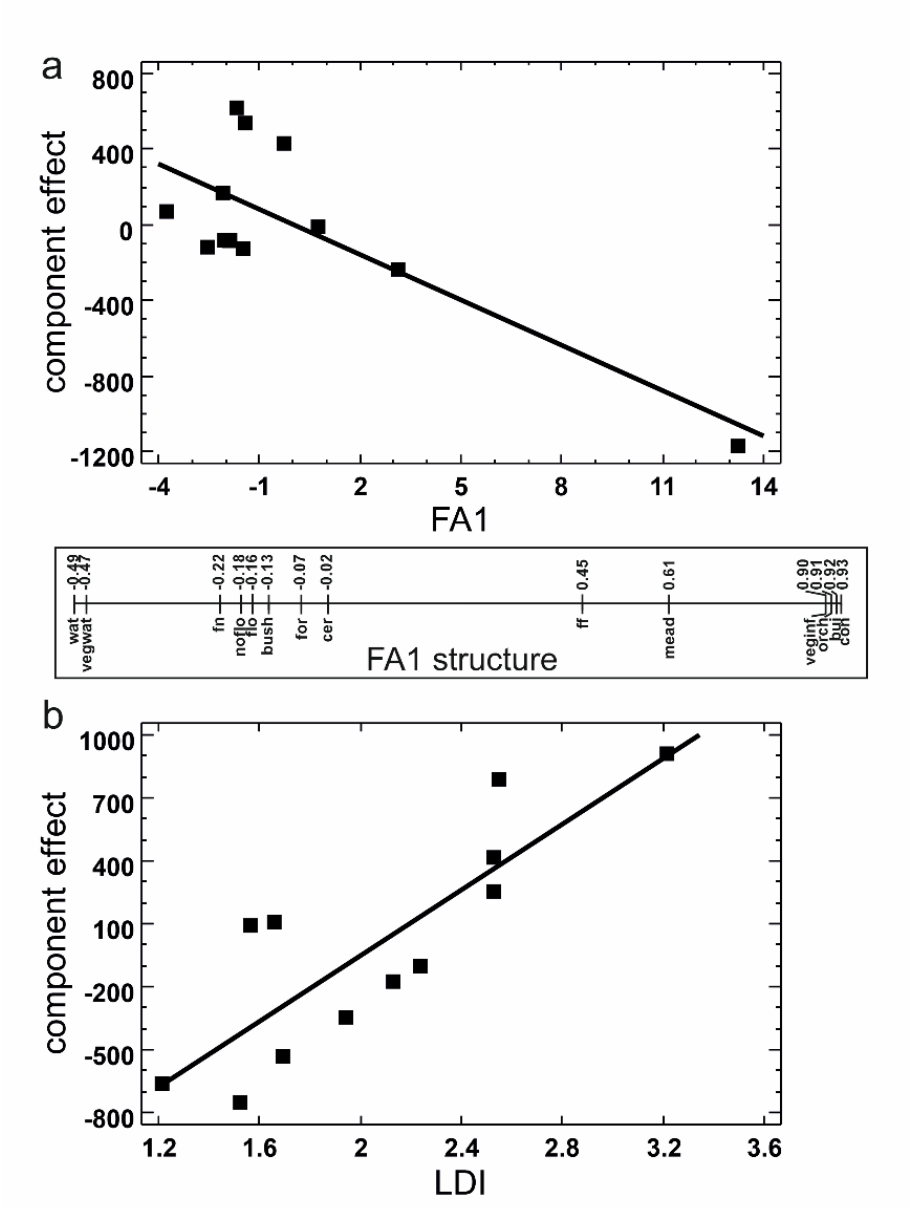

Figure S4. Results of the multiple regression analysis for the 500 m buffer: negative effects of (A) FA1 ($p = 0.011$) and positive effect of (B) LDI ($p = 0.003$) on the energetic value of pollen. The overall model including both variables was significant at $p = 0.008$ and explained 66% of the variability. The line shows the relative change in the predicted values of the energetic value of pollen when changing (A) FA1 or (B) LDI over their observed ranges. Each point (site) is then plotted by adding its residuals to a line. Note that the values on the y-axis are the residuals of the part of the model explained by another significant variable. The right side of the graph A shows the variables scores for 14 landscape units describing sites (*see Table S2 for a full description of the landscape units*) spread on the unitless FA1 axis.

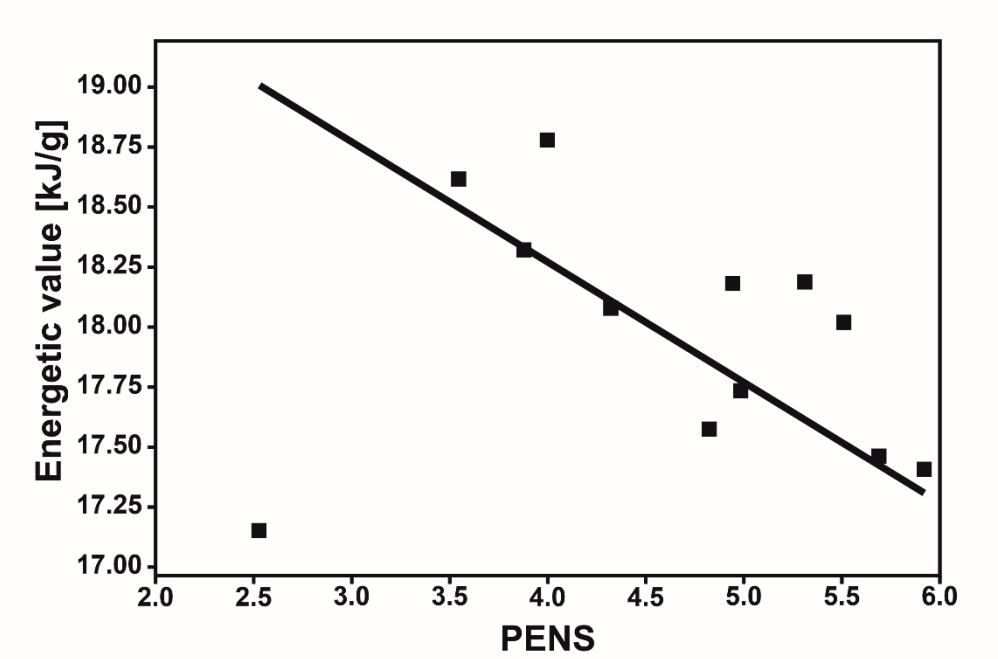

Figure S5. Results of the reduced major axis (RMA) regression: negative relationship between pollen diversity expressed as pollen effective number of species (PENS) and energetic value of pollen ($p = 0.009$) analysed in pollen provisions collected by *Osmia bicornis* for their larvae in 12 nests located in agricultural landscape.

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ARTYKUŁ III

Misiewicz, A., Zbrozek, M., Laskowski, R., Bednarska, A. J. Combined effects of PPPs on survival of the red mason bee *Osmia bicornis* – manuskrypt (w recenzji w Chemosphere).

Combined effects of insecticides on survival of the red mason bee *Osmia bicornis*

Anna Misiewicz[1*], Maryellen Zbrozek[2], Ryszard Laskowski[2], Agnieszka J. Bednarska[1]

[1]Institute of Nature Conservation, Polish Academy of Sciences, A. Mickiewicza 33, 31-120 Kraków, Poland

[2]Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

[*]Corresponding author: misiewicz@iop.krakow.pl

Abstract

Insecticides are widely used to control pests, but can also be toxic to ecosystem service providers, including bees. Moreover, their efficacy can increase if they are applied in mixtures. These mixtures can be intentional, e.g., created by farmers as tank mixtures (i.e., a mixture of different, individually formulated plant protection products (PPP) applied together in one application event or applied at short intervals), or unintentional, when exposure to a mixture results from bees foraging on different crops, each of which is treated with a different PPP. However, the combined toxicity of various PPPs remains largely unexplored, posing a potential threat to bees. Here, we assessed the interactive effects of five insecticides belonging to different chemical classes: organophosphate Dursban 480 EC (a.s. chlorpyrifos), two pyrethroids – Sherpa 100 EC (a.s. cypermethrin) and Karate Zeon 050 CS (a.s. lambda-cyhalothrin), neonicotinoid Mospilan 20 SP (a.s. acetamiprid), and sulfoximine Closer (a.s. sulfoxaflor)) applied topically as binary mixtures, on survival of *Osmia bicornis* females. Contrary to expectations, the experiment revealed either no interaction (in 'Dursban × Sherpa' experiment) or antagonistic interactions, particularly in mixtures of insecticide belonging to pyrethroids (Sherpa or Karate) with the one belonging to either neonicotinoids (Mospilan) or sulfoximines (Closer). Moreover, the mixture 'Karate × Closer' showed an antagonistic effect on the survival of *O. bicornis* already at field-relevant concentrations. The results suggest that mixtures of neonicotinoids and pyrethroids, commonly used nowadays, may be safer for *O. bicornis* than when the insecticides are applied individually. Such unexpected results emphasize the need for longer-term testing of cumulative toxicity effects via different exposure routes and on different bee species, as well as the need to consider the risk of exposure to multiple pesticides when assessing the safety of pesticides for bees.

Keywords: solitary bees, insecticide, mixture, interaction, antagonism

Highlights

- Binary mixture effects of insecticides on solitary bees were studied.
- Bees exposed to Dursban 480 EC, Karate Zeon 050 CS and Closer showed reduced lifespan.
- Antagonistic effects in binary mixtures of pyrethroid with either neonicotinoid or sulfoximine were observed.
- Mixtures of pyrethroids and neonicotinoids may pose less risk to *O. bicornis* than those insecticides applied individually.

1. Introduction

During the last decades, significant declines in wild bee diversity at local and regional scales have been observed (Biesmeijer et al., 2006; Hallmann et al., 2017; Zattara and Aizen, 2021). Although these declines are undoubtedly caused by a combination of different factors, including habitat loss, large-scale use of chemical pesticides and their metabolites have often been indicated as one of the main factors leading to a global decline in pollinators (Dudley and Alexander, 2017; Pisa et al., 2015; Uhl and Brühl, 2019; Woodcock et al., 2016). A total of 439 active substances are currently approved for use in plant protection of the various agricultural crops within the EU (European Commission, 2024) and their use in agriculture was 3.5 million tonnes in 2021, a twofold increase since 1990 (FAO, 2023). Among pesticides, insecticides in particular, which have increased substantially globally over the last decade (FAO, 2023), pose a serious threat to the environment as they can also affect beneficial insects such as pollinators (Brittain and Potts, 2011).

Pesticides are rarely found as individual chemicals in the environment. On the contrary, a variety of pesticide residues are frequently detected simultaneously (Tosi et al., 2018; Zioga et al., 2023). This is because they are often applied as mixtures, intentionally created by farmers to increase the efficacy of the treatment (i.e., tank mixes of several pesticides, often from different groups, applied together in one application event (Gazziero, 2015; Ngowi et al., 2007; Xu et al., 2008)) or sprayed consecutively at short time intervals (Jordaan et al., 2012; Tabashnik, 1989). Moreover, bees foraging on different crops are often exposed to many pesticides sprayed on those crops (Heys et al., 2016). A recent study has shown that the detection of mixtures of compounds in the same matrix (either floral pollen or nectar) from the same field was more the rule than the exception, and often these detections did not correspond with the pesticides recently applied (Zioga et al. 2023). Thus, the toxicity of mixtures of pesticides to bees has become an important safety concern (Williams et al., 2023) but hasn’t been introduced yet into ecological risk assessment (ERA) or registration procedures of Plant Protection Products (PPPs) (EFSA et al., 2023). The current ERA of pesticides in Europe considers almost exclusively single applications of single PPPs on a single crop (Topping et al., 2020). More specifically, the ERA includes only the “mixture” in a single PPP, which is a formulation of one or more active substances and additives (surfactants, penetrant enhancers, spreaders, stickers, UV blocking agents, and/or co-solvents) used to optimize the efficacy and stability of the active substances (Mesnage and Antoniou, 2018) and thus improve the PPP's properties such as solubility for example. If at all, PPP applications with one or more PPPs at

the same time are only considered in rare cases where mixtures of several PPPs are specifically registered as such and listed on the label with a clear name and dose rate.

Pesticides in mixtures can potentially interact with each other, causing both lethal and sublethal effects in bees (Boff et al., 2021; Brandt et al., 2020; Heys et al., 2016). The effects of pesticide mixtures can be more harmful to organisms than the sum of effects of individual substances due to their synergistic effects, as found, for example, in the case of combined exposure of *Osmia bicornis* to clothianidin and propiconazole (Sgolastra et al., 2018). Also, the toxicity of a combination of chlorothalonil (which did not caused mortality when applied alone) and imidacloprid appeared to be 23.9 times higher for *Apis mellifera* and 83.4 times higher for *Partamona helleri* than imidacloprid used alone (Tomé et al., 2017). Another type of non-additive effect of pesticides in mixtures is antagonism, where combined toxicity is lower than the sum of each pesticide's toxicity, as was found, for example, in *A. mellifera* exposed to a mixture of sterol biosynthesis inhibiting fungicides with tau-fluwalinat (at lowest dose) (Johnson et al., 2013). However, antagonistic effects have been reported much less frequently than synergistic (Carnesecchi et al., 2019).

The mixture effects of PPPs in bees are poorly recognized; for example, interactions between different insecticides have only been studied in about 6% of pesticide-related experiments on honey bees (Benuszak et al., 2017). Carnesecchi et al. (2019) showed that out of 957 publications, only 14 were on the effects of mixtures of pesticides. The major part (10 articles) focused on *A. mellifera*, while four articles included *Bombus* spp. and *Osmia* spp. Also, a recent comprehensive overview of the combined pesticides' toxicity in bees by Tosi et al. (2022) indicated that most effects of binary pesticide combinations were tested on the western honey bee *A. mellifera*, which is a standard species in ecological risk assessment of pesticides (Committee et al., 2021). Honey bees, however, are not the only insect pollinators that contribute to biodiversity by providing critical pollination services (Brittain et al., 2013). Non-*Apis* species can also serve as important pollinators of crops, either as wild or managed populations (Garibaldi et al., 2014; Garibaldi et al., 2013; Kremen et al., 2002), but despite their important role in the environment (Artz and Pitts-Singer, 2015; Heard et al., 2017; Kopit et al., 2022; Sgolastra et al., 2018; Losey and Vaughan, 2006) they have received much less attention. Non-*Apis* bees may, however, respond to pesticides and their mixtures differently because of differences in physiology and ecology (Brittain and Potts, 2011; Tomé et al., 2017). This is supported by a meta-analysis comparing the pesticide sensitivity of the honey bee with that of other bee species (Arena and Sgolastra, 2014), including *Osmia spp.*, which has been identified as a suitable model species for ecological risk assessments (EFSA et al., 2023). Both single and

multiple pesticide exposures in bees can occur through multiple routes, such as nectar (Krupke et al., 2012), pollen (Tosi et al., 2018) or water (Samson-Robert et al., 2014), but in addition to oral exposure, exposure through direct contact with the sprayed pesticide(s) is also possible, especially in species foraging in crops with high bee attractiveness, such as oilseed rape. Some pesticides, for example hydrophobic insecticides, including pyrethroids, are on average three times more toxic to bees through contact than through oral exposure, whereas most hydrophilic pesticides, such as sulfoxaflor, are more toxic via oral exposure, sometimes reaching 11–13 times higher toxicity (Sanchez-Bayo and Goka, 2014).

Here, we assessed the combined effects of binary mixtures of PPPs with different active substances (a.s.) with different modes of action (Sparks and Nauen, 2015), on the survival of *O. bicornis*, which is a native pollinator across Europe, northern Africa, and western Asia (Amiet et al., 2004). The effects of topical exposure of bees on the field-relevant formulations of three binary mixtures of PPPs were studied: (i) organophosphate Dursban 480 EC (a.s. chlorpyrifos) with pyrethroid Sherpa 100 EC (a.s cypermethrin) (Dursban × Sherpa), (ii) neonicotinoid Mospilan 20 SP (a.s. acetamiprid) with Sherpa 100 EC (Mospilan × Sherpa), and (iii) pyrethroid Karate Zeon 050 CS (a.s. lambda-cyhalothrin) with and sulfoximine Closer (a.s. sulfoxaflor) (Karate × Closer). In agricultural environments, bees are likely to be exposed simultaneously to selected binary mixtures as these PPPs are commonly applied to various crops (Kortenkamp and Faust, 2018; Levine and Borgert, 2018; Mu et al., 2022).

Methods

2.1. Study organism

Osmia bicornis cocoons were purchased from a local supplier (BioDar, Poland) in February 2020 and in March 2021 and stored at 4°C in darkness until use. In spring, the largest cocoons (expected to be females) were transferred to large plexiglas boxes (46×30×17 cm) with air flow provision from the top and were left to hatch at temperature 20°C, 60±5% relative humidity (RH) under 16:8 hour light:dark (L:D) regime until emergence. The emergence was checked two times a day, and the emerged males (if present) were discarded. The emerged females were fed *ad libitum* with sucrose solution 33% (w/w) provided in 2-ml Eppendorf tubes with cotton wool inside the tube and with a small square-cut yellow sponge-cloth provided around the tube (Fig. S1A) to ensure the bees located the feeder quickly. At least 4-day-old unmated females were used in experiments to avoid loss of portion of the population early during husbandry (Robinson et al., 2017).

141 *2.2. Insecticides*

142 The field-relevant formulations of PPPs available on the market, containing active
143 substances with different modes of action, were used: Dursban 480 EC (Dursban; 44.86%
144 chlorpyrifos a.s., Dow AgroSciences, Poland), Sherpa 100 EC (Sherpa; 10.76% cypermethrin
145 a.s., Chemirol, Poland), Mospilan 20 SP (Mospilan; 20% acetamiprid a.s., Sumi Agro, Poland),
146 Karate Zeon 050 CS (Karate; 4.81% lambda-cyhalothrin a.s.; Syngenta, Poland) and Closer
147 (11,3% sulfoxaflor a.s., Dow AgroSciences, Poland).

148 Stock solutions of each PPP were prepared in 100 ml of 0.01% Triton X-100 (used to
149 facilitate the adhesion of the solution to the bee body) as 10 × Recommended Application
150 Concentration (RAC) given by their manufacturers for spray application in oilseed rape. Then,
151 stock solutions were diluted in 0.01% Triton to achieve the desired range of concentrations of
152 individual insecticides and their mixtures (Table 1). In the case of Dursban, Sherpa and
153 Mospilan, the ranges of concentrations were chosen taking into account their individual toxicity
154 to *O. bicornis* females after topical application found by Mokkapati et al. (2021). Literature
155 data on the LD_{50s} for *O. bicornis* and *A. mellifera* (Arena and Sgolastra, 2014; Lewis et al.,
156 2016) and for *A. mellifera* and *Bombus terrestris* (Bacci et al., 2018) were used to select
157 concentrations of lambda-cyhalothrin and sulfoxaflor, respectively, and then to recalculate
158 them into concentrations for Karate and Closer, respectively, and expressed as a multiple (or
159 fraction) of the RAC.

160 *2.3. Experimental design*

161 Two full-factorial experiments with binary mixtures of PPPs were conducted in 2020:
162 'Dursban × Sherpa' and 'Mospilan × Sherpa' and one full-factorial experiment was conducted
163 in 2021: 'Karate × Closer'. The following five concentrations were used: 0, 0.2, 0.4, 0.7,
164 1 × RAC for Dursban (i.e., 0, 192, 384, 672, and 960 ng/µL for chlorpyrifos as a.s.) and 0, 0.25,
165 0.5, 1 and 5 × RAC) for Sherpa (i.e., 0, 25, 50, 100 and 500 ng/µL for cypermethrin as a.s.) in
166 the experiment 'Dursban × Sherpa'; 0, 0.04, 0.2, 1 and 5 × RAC for Mospilan (0, 3.2, 16, 80
167 and 400 ng/µL for acetamiprid as a.s.) and Sherpa (0, 4, 20, 100 and 500 ng/µL for cypermethrin
168 as a.s) in the experiment 'Mospilan × Sherpa'; 0, 0.2, 1, 5, and 25 × RAC for Karate (0, 5, 25,
169 125, and 625 ng/µL for lamda-cyhalothrin as a.s.) and Closer (0, 24, 120, 600, and 3000 ng/µL
170 for sulfoxaflor as a.s.) in the experiment 'Karate × Closer' (Table 1). Triton X-100 at 0.01%
171 was used as a control. The concentrations of active substances were confirmed in selected
172 treatments used for the experiment. The chemical analyses were done by the certified external
173 contractor – the Regional Experimental Station of the Institute of Plant Protection, National

Research Institute in Białystok, Poland, using LC-MS/MS or GS-MS/MS techniques. The measured concentrations of chlorpyrifos, cypermethrin and acetamiprid were on average 116% of their nominal concentrations. For lambda-cyhalothrin and sulfoxaflor, the measured concentrations were higher than expected, with an average of 166% of nominal concentrations, but still represented the assumed geometric series. Moreover, similar concentrations were measured for the same insecticide regardless of whether it was applied individually or in a mixture. The results of the chemical analyses are presented in Table S1.

In two experiments, 'Dursban × Sherpa' and 'Karate × Closer', 20 females per treatment were used, and in the experiment 'Mospilan × Sherpa' 30 females per treatment were used. The bees were treated individually using a topical application of 1 µl of the test solution (either insecticide(s) solution or 0.01% Triton X-100) on the dorsal thorax using a Hamilton micro-syringe with a dispenser (Fig. S1B). About one hour before treatment, bees were taken from the cages, placed in glass Petri dishes (5 bees/dish) and then placed at 4°C for approximately 20 min to limit their mobility and ensure proper application of test solution (i.e., prevent the bees from spreading the solution to the neck or wing hinges). The exposed bees were then transferred to plastic boxes (30×19.5×20.5 cm) and moved to the climatic chamber (20±2°C, 60±5% RH, 16:8 L:D) (Fig. S1CD). Despite of being treated individually, the bees were group-housed in boxes and were fed *ad libitum* with 33% (w/w) sucrose solution placed in Eppendorf tubes as described above (Fig. S1A). An additional control treatment with bees not exposed at all was used in each experiment to control for the possible effect of 0.01% Triton X-100. The survival of bees was checked daily until all bees died. During daily checks, the food was replenished. The dead bees were removed from the boxes consecutively.

2.4. Data analysis

Since the normal distribution of data on life times was not met (Shapiro Wilk W test), the effects of PPPs and their interaction on life times of *O. bicornis* was tested using two-way PERMANOVA with 9999 permutations in Past program version 4.08 (Hammer et al., 2001). To visualize the effects in 3D graphs, a general linear models (GLMs) analysis was used (Statgraphics Centurion program, version 19.4.04, Statgraphics Technologies Inc.). Bees that escaped or were found covered with sucrose solution as well as those with exceptionally long lifespans were removed from PERMANOVA as outliers (25 bees (from among 509) were excluded in 'Dursban × Sherpa' experiment, 35 bees (from among 781) were excluded in 'Sherpa × Mospilan' experiment and 6 bees (from among 519) were excluded in 'Closer × Karate' experiment) but were included as right-censored in the Kaplan-Meier

survival analyses. Kaplan-Meier survival analysis was used to create survival curves of the different treatment groups and to individually compare treatments of interest using the Log-rank test (Statgraphics Centurion program, version 19.4.04, Statgraphics Technologies Inc.). The sensitivity of bees to each treatment was additionally expressed as the median lethal time (LT_{50s}) estimated from the survival curves.

3. Results

3.1. Survival of Osmia bicornis after exposure to Dursban × Sherpa

The survival curves of bees in the two control groups (treated with 0.01% Triton and not treated at all) did not differ ($p = 0.22$), indicating that Triton did not influence survival. Therefore, both controls were combined for further analyses, resulting in $LT_{50s} = 29 \pm 3.2$ days.

Two-way PERMANOVA indicated a significant negative effect of Dursban ($p \leq 0.001$) and Sherpa ($p = 0.015$), with no interaction ($p = 0.7$; Fig. 1A) on bees life time.

Very high toxicity was observed for Dursban at 0.7 × RAC and 1 × RAC (Table 2), with LT_{50s} of 1 day for both concentrations, regardless of whether the bees were additionally exposed to Sherpa. The survival of bees exposed to Sherpa alone was higher, with LT_{50s} from 10±3.1 to 30±1.7 days, depending on concentration (Table 2). Sherpa alone did not affect bee survival at the studied concentrations, except for the negative effect for Sherpa alone at 0.25 × RAC: Kaplan-Meier survival analysis showed significantly lower survival of bees at 0.25 × RAC Sherpa compared to all other concentrations of this insecticide (0.5, 1, 5 × RAC, $p \leq 0.001$) and to control ($p = 0.0002$; Table 2). However, the additional presence of Dursban at both 0.2 × RAC and 0.4 × RAC in a treatment did not modify the survival of Sherpa-exposed bees, as no differences in survival curves were found between Sherpa-exposed bees for those two concentrations of Dursban ($p \geq 0.13$). When Dursban was applied alone, the bees had significantly lower survival at two highest doses (0.7 or 1 × RAC) compared to other doses (0.2 and 0.4 × RAC, $p < 0.001$) and to control ($p < 0.001$) (Fig. 2A). When Dursban was applied in a mixture with the highest concentration of Sherpa (5 × RAC), significantly lower survival of bees was found at the two higher concentrations of Dursban (0.7 and 1 × RAC) compared to both 0.2 × RAC ($p \leq 0.09$) and the control ($p \leq 0.03$), and there was no difference between 0.4 × RAC and 0.7 × RAC of Dursban ($p = 0.4$; Fig. 2B).

3.2. Survival of Osmia bicornis after exposure to Mospilan 20 SP C × Sherpa 100 EC

Survival in the two control groups differed ($p \leq 0.001$), indicating that Triton might have influenced survival, so only the treatment with Triton (LT_{50s} of 25±8.2 days) was included as a control for further statistical analysis.

PERMANOVA of life times detected a significant effect of Sherpa ($p = 0.019$) and an antagonistic interaction with Mospilan ($p = 0.001$) (Fig. 1B).

Kaplan-Meier analysis did not indicate a difference in survival curves between bees treated with Sherpa or Mospilan alone ($p \geq 0.1$), but the presence of Mospilan at 0.2 × RAC modified the survival of bees which were simultaneously exposed to Sherpa: those exposed to Sherpa at 0.2 × RAC lived longer ($LT_{50s} = 44$ days) than control bees ($LT_{50s} = 36$ days, $p \leq 0.001$) and than those exposed to 0.04 × RAC ($LT_{50s} = 37$ days; $p = 0.012$), 1 × RAC ($LT_{50s} = 28$ days; $p \leq 0.001$) and 5 × RAC ($LT_{50s} = 33$ days; $p = 0.015$). Mospilan at 1 × RAC did not affect the survival negatively in any combination with Sherpa but the exposure of bees to both Mospilan and Sherpa at their highest concentrations (5 × RAC) unexpectedly had a positive effect on bees' survival ($LT_{50s} = 37$ days): bees survived longer than those exposed to Mospilan alone at 5 × RAC ($LT_{50s} = 30$ days, $p = 0.037$) and similarly to those exposed to Sherpa alone at 5 × RAC ($LT_{50s} = 39$ days, $p = 0.08$). As mentioned, Mospilan applied individually did not affect the survival of bees ($p = 0.8$), although LT_{50s} varied from 26 to 40 days (Table 3, Fig. 2C), but Mospilan applied at its highest concentration affected survival of bees at 0.04 × RAC Sherpa treatment ($p = 0.01$; Fig. 2D).

3.3. Survival of Osmia bicornis after exposure to Karate Zeon 050 CS × Closer

Survival in the two control groups did not differ ($p = 0.153$), indicating that Triton did not influence survival. Thus, both controls were combined, resulting in $LT_{50s} = 19$±0.27 days.

Two-way PERMANOVA showed that both Karate and Closer as well as their interaction had a significant effect on *O. bicornis* survival (Karate: $p \leq 0.001$; Closer: $p \leq 0.0001$; interaction: $p \leq 0.0001$; Fig. 1C).

Kaplan-Meier analysis indicated that both Karate and Closer applied individually decreased the survival of bees ($p \leq 0.00001$ for each PPP) and survival was lower at all concentrations in comparison with control ($p \leq 0.00001$) as well as the most seriously affected at higher concentrations (LT_{50s} equaled 3±1.0 days at 25 × RAC for Karate and 1 day at both 5 × RAC and 25 × RAC for Closer, in comparison with 19±0.3 days for control). Generally, median lethal times (LT_{50s}) at the higher concentrations of PPPs (5 × RAC or 25 × RAC), whether alone or in a mixture, dropped to 2 days and 1 day, respectively. There was no monotonic relationship

between Karate concentration and mortality when Karate was applied alone – at the first three consecutive concentrations (0.2, 1, and 5 × RAC) the LT_{50s} were identical (Table 4) and the survival curves did not differ ($p \geq 0.68$). Closer exhibited even more unexpected results when applied alone: the bees treated with 0.2 × RAC had significantly higher survival compared to the control ($p = 0.004$; Table 4; Fig. 2E). However, the combination of both PPPs at 1 × RAC resulted in an unusually high LT_{50s} (26 days) – bees survived significantly better than the control ones ($p < 0.001$, Table 4) and better than those in treatments with each PPP applied alone ($p \leq 0.001$ and $p \leq 0.001$, for Karate and Closer, respectively; Table 4), indicating an antagonistic interaction between Karate and Closer. The interaction detected between Karate and Closer was also seen for Karate at 25 × RAC when applied with Closer at or below the RAC, resulting in similar survival of bees to those exposed to 25 × RAC Karate alone ($p \geq 0.14$; Fig. 2F). Also, when Karate was applied at the 1 × RAC, the application of Closer at concentrations at or below the RAC resulted in an antagonistic effect on mortality ($p \leq 0.000004$; Table 4). However, no interaction was seen when Karate was used in combination with Closer at 25 × RAC ($p = 0.2$), indicating that the negative effect on bee survival was dominated by Closer.

Discussion

Because antagonistic effects have been reported much less frequently than synergistic (Carnesecchi et al., 2019) for binary mixtures of pesticides, and considering different modes of action of the studied PPPs, we hypothesized their synergistic effects on the survival of *O. bicornis* at gradually increasing concentrations. In contrast, antagonistic interaction occurred between Sherpa and Mospilan (at concentrations higher and lower than RACs) and between Karate and Closer (specifically when one or both PPPs were applied at concentrations near RACs). No statistically significant interaction between Dursban and Sherpa was found, mostly due to the high toxicity of Dursban already at concentrations much lower than RAC. In each of the studied binary mixtures, two insecticides with different modes of action (i.e., acting on different pathways or affecting different mechanisms crucial for insect functioning and survival) were combined (Sparks and Nauen, 2015; Stenersen, 2004). Both mixtures in which antagonistic reactions occurred, contained one insecticide that belongs to pyrethroids (Sherpa or Karate), which modify the function of neuronal membrane-bound voltage-gated sodium channels in insects, leading to disruptions in the transmission of electrical signals within the nervous system (Soderlund, 2010), and the other one belonging to either neonicotinoids (Mospilan) or sulfoximines (Closer). Although technically active substances of Mospilan and

Closer belong to different groups, they act in a similar manner. Neonicotinoids act as agonists on insects' nicotinic acetylcholine receptor (nAChR), disrupting the initiation of electric signals in postsynaptic neurons (Seifert, 2014). Those insecticides replace acetylcholine at the agonist site of the receptors, causing the channels to remain open, which leads to lethal overstimulation of neuronal activity within minutes (Casida, 2018). The sulfoximines are a new class of insecticides created as an alternative to neonicotinoids (Bacci et al., 2018). They are also nAChR agonists, but structural differences (structure-activity relationships) let them to be classified separately from neonicotinoids (Sparks et al., 2013).

In the experiment 'Sherpa × Mospilan', the interaction between those two PPPs showed that Mospilan at 0.2 × RAC (i.e., 16 ng/µL for acetamiprid) enhanced the survival of Sherpa exposed bees at 0.2 × RAC (i.e., 20 ng/µL for cypermethrin). Similarly, Mospilan at the highest dose (5 × RAC, i.e., 400 ng/µL for acetamiprid) enhanced survival of Sherpa exposed bees (at 5 × RAC, i.e., 500 ng/µL for cypermethrin). After topical exposure, the 24-h LC_{50s} for cypermethrin applied in Sherpa was 3330 ng/µL for *O. bicornis* (Mokkapati et al. 2021) and for cypermethrin applied as the active substance it was 2.27 ng/µL for *A. mellifera* (Mazi et al., 2020). This suggests that either cypermethrin is much less toxic to *O. bicornis* than to *A. mellifera* or much more toxic as an active substance than in a formulation. The lack of negative effects of acetamiprid on bees' survival in our experiment within the tested range supports the previous findings in which low toxicity of this insecticide was observed in *O. bicornis* exposed topically to Mospilan (the 24-h LC_{50s} for acetamiprid applied in Mopsilan 20 SP was 4090 ng/µL in Mokkapati et al, 2021), in *Tetragonisca angustula* (a small eusocial stingless bee) orally exposed to Mospilan (Jacob et al., 2019), as well as in *Osmia corniforis* exposed topically to Assail 30SG (acetamiprid 30%) (Biddinger et al., 2013). Although there are no other studies in which the combined effects of Sherpa and Mospilan or their active substances (i.e., cypermethrin and acetamiprid, respectively) were studied on bees, a few studies confirmed antagonistic interactions between insecticides from the same classes as those studied here. For example, Li et al. (2023a) showed the antagonistic effect of a mixture of neonicotinoid thiamethoxam and pyrethroid esfenvalerate (formulated pesticides) on the survival of orally exposed *A. mellifera*. The authors tested the effect of thiamethoxam also in combinations with three other pyrethroids (zeta-cypermethrin, cyfluthrin, and permethrin), but in these cases the effects were synergistic. Wang et al. (2021) also tested binary mixtures of a neonicotinoid (Belay 50 WDG; clothianidin a.s.) with two pyrethroids (either Baythroid XL 1 EC; beta-cyfluthrina a.s. or Declare; gamma-cyhalothrin a.s.) in orally exposed *A. mellifera*. Two days

after the exposure, each combination exhibited antagonistic interactions in their toxicological effects on honey bees survival (Wang et al., 2021).

The antagonistic effect was also observed for the mixture 'Karate × Closer'. Combinations of these compounds increased the survival of *O. bicornis* compared to controls when both pesticides were used at their RACs. As the RAC is likely the concentration used by farmers in the field, these results are intriguing considering that topical exposure to the combination of the two pesticides at these concentrations improved survival compared to either pesticide used alone. Sulfoxaflor (a.s. in Closer) was recognized as very hazardous to honey bees and buff-tailed bumblebees by the European Food Safety Authority (EFSA et al., 2020). Restrictions on its use have been introduced in Europe and the USA (OJEU, 2022; US EPA, 2019). In our study, the two highest applied doses of Closer (5 × RAC and 25 × RAC) were very toxic for bees as half of the tested bees died already one day after applying these doses of the insecticide. However, at the lower dose (0.2 × RAC) we observed unexpected results: treated bees had significantly higher survival compared to the control, although their LT_{50s} were similar (21±1.5 days vs 19±0.3 days). This indicates a hormesis, i.e. a situation in which low doses of a stressor can stimulate biological processes (Cutler and Rix, 2015). This phenomenon was also observed in ground-nesting bees exposed to nesting substrates contaminated with imidacloprid – the hormetic responses were observed in *Megachile rotundata* development speed and body mass (Anderson and Harmon-Threatt, 2019).

In the case of Karate applied alone, reduced survival compared to the control was noted, especially at the highest concentration. At the first three consecutive concentrations (0.2, 1, and 5 × RAC) of Karate alone, the LT_{50s} values were identical, and the survival curves did not differ between each other but differed from control. Another study has also shown negative effects of this insecticide on bees (Deepika et al., 2022). Contact toxicity (filter paper with the insecticide) of lambda-cyhalothrin (5EC, 0.6 mL/L) caused a mortality of 46% and 68% to *Apis cerana indica* and *Tetragonula iridipennis*, respectively, 24 hours after exposure (Deepika et al., 2022). On the other hand, it seems that Closer can cause hormesis alone, and this effect is amplified when combined with Karate. There is ample evidence that hormesis can occur when insects are exposed to low doses of insecticides, and while most of this evidence focuses on insect pests, it has also been observed in beneficial insects such as bees (Cutler and Rix, 2015). For example, hormesis has been observed in solitary bees exposed to soil contaminated with imidacloprid, however, the fundamental mechanisms remain unknown (Anderson and Harmon-Threatt, 2019). In the case of antagonism between 'Closer × Karate', to the best of our knowledge, our

study is the first to investigate interactions between sulfoximines and pyrethroids. The effect of toxins on insect survival could be associated with the induction of detoxification enzymes, including cytochrome P450 monooxygenases (CYP450s) (Cutler and Rix, 2015). Studies indicate that CYP450 expression can influence sulfoxaflor resistance in insect pests (Li et al., 2023b; Wang et al., 2022; Watson et al., 2021), and may play a role in reducing the toxicity of certain insecticides in non-target organisms like bees (Johnson et al., 2006). Furthermore, it has been demonstrated that CYP450 expression reduces lambda-cyhalothrin toxicity in honey bees (Johnson et al., 2006). CYP450s involved in pyrethroid (and other pesticides) metabolism can be induced by pyrethroids themselves, altering detoxification abilities (Hernández et al., 2017). Therefore, it is possible that the combination of Closer and Karate at the specific doses for which antagonism were observed may strongly induce CYP450 upregulation, enhancing the detoxification of insecticides. However, this effect may not be sustainable at higher insecticide concentrations, leading to faster mortality. In the cases where hormesis was observed, it is possible that the strongly increased CYP450 upregulation assists in the detoxification of some natural toxins that are present in the bees, as well as detoxification of the insecticides themselves. For instance, topical application of 0.2 × RAC Closer alone could upregulate CYP450 enough to detoxify other natural toxins and improve survival, but this effect may not occur when higher Closer concentrations are used because the toxicity of the insecticide becomes too strong. However, when combined with Karate and its additional CYP450 upregulation potential, the ability to detoxify natural toxins and the insecticides may potentially be restored; this could explain the hormesis observed when both insecticides were applied at the RAC. Nevertheless, such an effect requires more detailed studies.

A very high toxicity of Dursban was found when it was applied both at and below the concentration recommended by the producer (i.e., 1 and 0.7 × RAC). This was visible when Dursban was applied individually as well as in combination with Sherpa at all tested concentrations, where Dursban caused 100% mortality of female bees within one day. Such high toxicity of Dursban was also found in *O. bicornis* males; our previous studies showed that it killed all males within one day already at 0.25 × RAC (Misiewicz et al., 2023). Many studies have confirmed the negative effects of chlorpyrifos (a.s. in Dursban) on the survival of bees (e.g., *Scaptotrigona bipunctata*, *Tetragonisca fiebrigi*, *T. angustula*, *A. mellifera* and *B. terrestris*) (Dorneles et al., 2017; Leite et al., 2022; Pervez and Manzoor, 2021; Reid et al., 2020, respectively). Although chlorpyrifos usage was prohibited in the European Union in 2020 (OJEU, 2020) and then also in the USA in 2022 (US EPA, 2021), chlorpyrifos-based PPPs are

still used in many countries around the world (Nai et al., 2017; Onwona-Kwakye et al., 2020; Urlacher et al., 2016), potentially threatening pollinating insects. Sherpa showed much lower toxicity than Dursban, but significant effect of Sherpa alone on bee survival was noticeable, but only in one experiment in which Sherpa was used. Surprisingly, in the experiment 'Dursban × Sherpa', females exposed to the lowest concentration of Sherpa, i.e., 0.25 × RAC, lived significantly shorter than those from control and those exposed to higher tested concentrations. One may hypothesize that the 0.25 × RAC Sherpa paradoxically could induce a stress mechanism in the bees that could have been more harmful than the direct toxic effects of higher doses. On the other hand, similar effect was not observed in the second experiment, although similar concentration of Sherpa (0.2 × RAC) was used.

Conclusion

Our findings revealed that the studied PPPs either interacted antagonistically or that there was no interaction between them. Antagonistic interactions were observed in 'Sherpa × Mospilan' and 'Karate × Closer' mixtures, even at concentrations above those recommended for field use. Notably, these interactions occurred in two mixtures of insecticides with different modes of action, but always when the one affecting neuronal membrane-bound voltage-gated sodium channel was combined with the one being nAChR agonist, highlighting the complexity of pesticide interactions with non-target organisms. Our results suggest that when either insecticide is applied at the recommended concentration, the addition of the other at its RAC or lower concentrations is inconsequential or even beneficial for the survival of *O. bicornis*. However, this does not exclude the possibility that Sherpa, Mospilan or their interaction, as well as Closer, Karate or their interaction, have sublethal effects on *O. bicornis*, nor the possibility that other organisms would be negatively affected by the application of these insecticides. This may be supported by the fact that our results for *O. bicornis* are opposite to those found for the bumblebee *B. terrestris* by Gill et al. (2012) who showed that combined oral exposure to neonicotinoid and pyrethroid increased the propensity of colonies to fail. Due to insect resistance to single insecticides, farmers have already started using mixtures of insecticides to achieve high efficacy and slow down the development of pests' resistance. Our results emphasize the need for further research to better understand the effects of PPPs combinations on pollinators like *O. bicornis* and their general effect on the health of wild bees, which could have far-reaching consequences for pollinator protection in an agricultural landscape.

Founding

This study was supported by the National Science Centre, Poland (NCN grant number UMO-2017/26/D/NZ8/00606) and in part by the EcoStack project (Grant Agreement 773554-H2020-SFS-2016-2017/H2020-SFS-2017-2). Thanks to the EcoStack grant it was possible to extend the number of studied mixtures.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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FIGURES

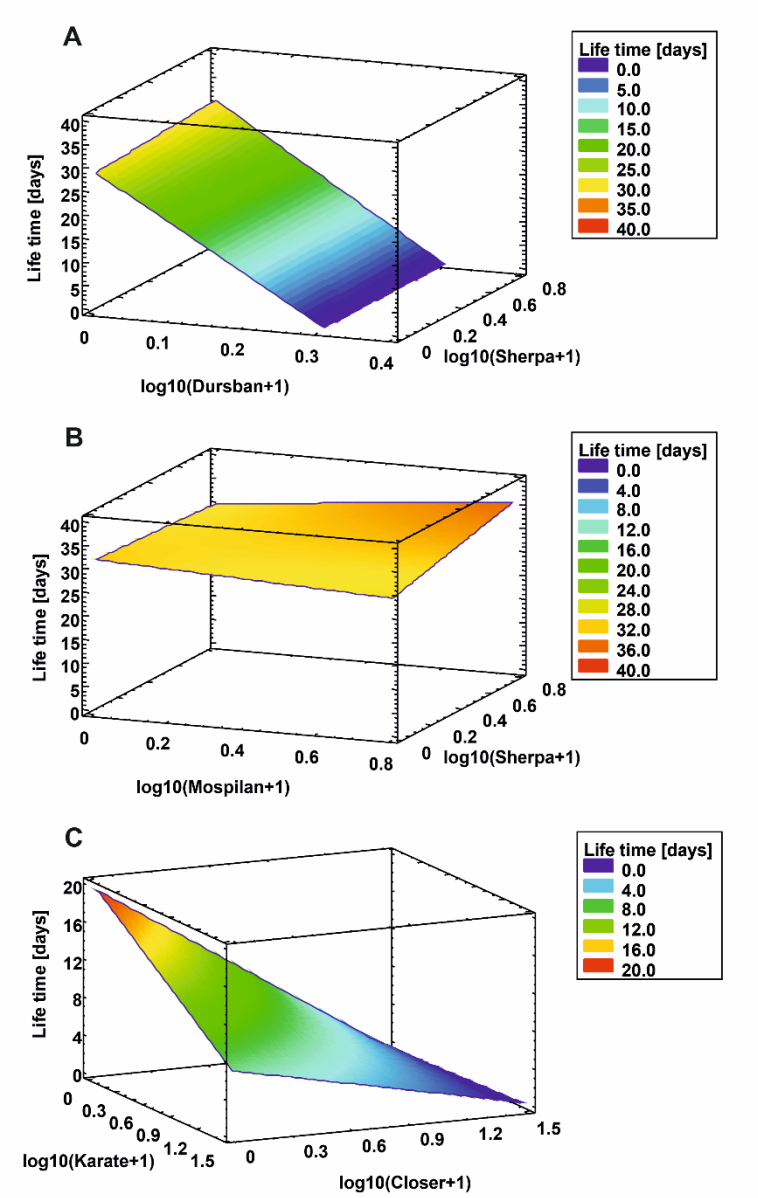


Figure 1. Estimated response surfaces for the effect of **(A)** Dursban 480 EC ($p \leq 0.001$) and Sherpa 100 EC ($p = 0.015$), **(B)** the interaction ($p \leq 0.001$) between Sherpa 100 EC ($p = 0.019$) and Mospilan 20 SP ($p = 0.172$), and **(C)** the interaction ($p \leq 0.001$) between Karate Zeon 050 CS ($p \leq 0.001$) and Closer ($p \leq 0.001$) on life time [days] in full factorial experiments with female *Osmia bicornis* exposed topically to insecticide(s).

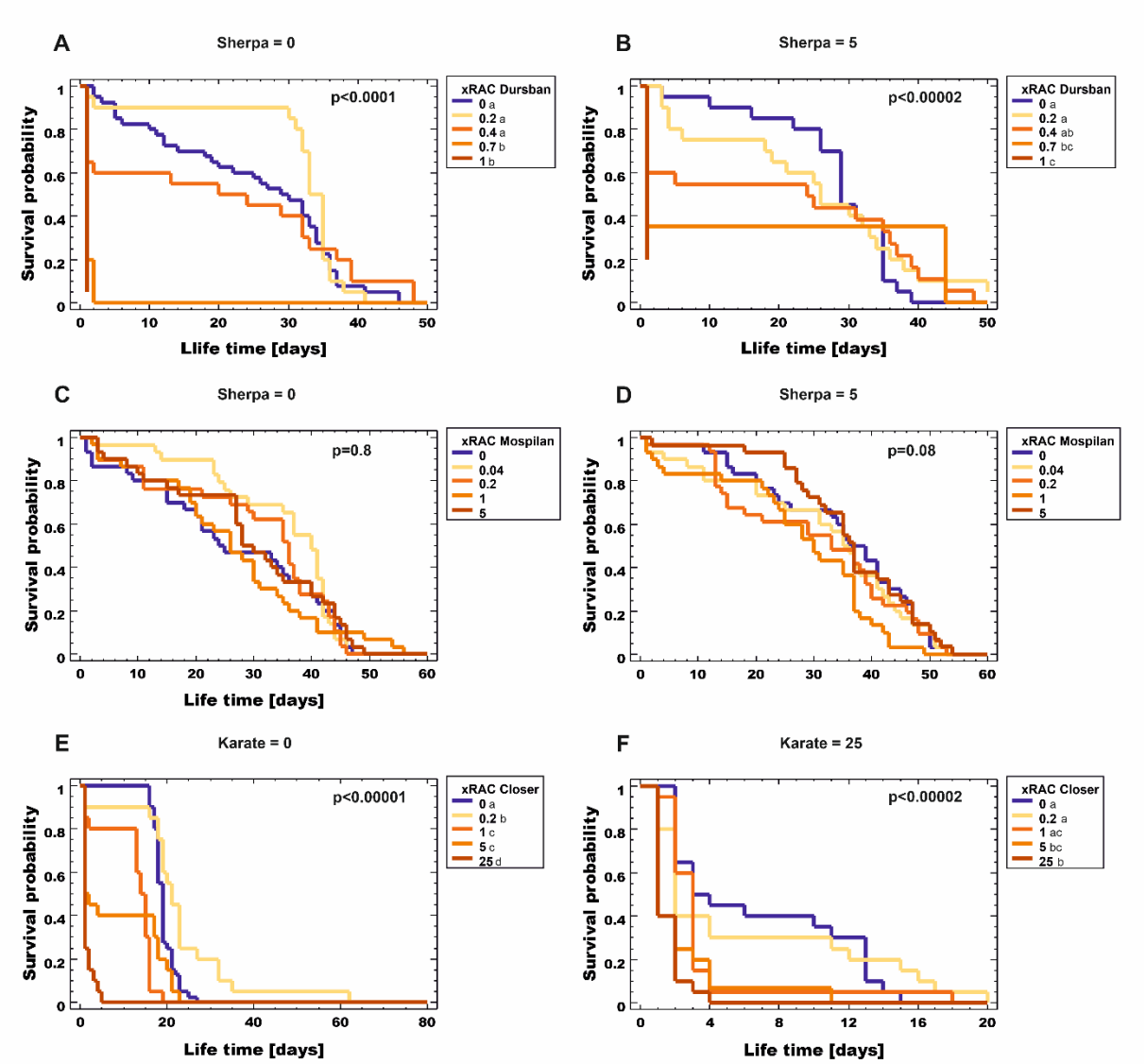


Figure 2. Survival curves for *Osmia bicornis* when no (left column) or maximum (right column) concentration of one insecticide was used in combination with the incremental concentrations (expressed relative to the recommended application concentration, RAC) of the other insecticide: (**A**) Dursban 480 EC used alone, (**B**) Sherpa 100 EC applied at maximum concentration, (**C**) Mospilan 20 SP used alone, (**D**) Sherpa 100 EC applied at maximum concentration, (**E**) Closer used alone, and (**F**) Karate Zeon 050 CS applied at maximum concentration. Survival curves sharing the same letter placed next to each concentration do not differ significantly.

TABLES

Table 1. Nominal concentrations of five Plant Protection Products: Sherpa 100 EC, Dursban 480 EC, Mospilan 20 SP, Karate Zeon 05 CS, and Closer expressed relative to the recommended application concentration (× RAC) and as concentrations of their active substances [ng/µL], i.e., cypermethrin, chlorpyrifos, acetamiprid, lamda-cyhalothrin, and sulfoxaflor, respectively, used in a full factorial experiment to study their effects on survival of female *Osmia bicornis* bees after topical exposure. Because each bee received 1 µL of the solution, the reported concentrations correspond to doses expressed in nanograms of the active substance per bee (ng/bee).

Treatment	PPP		Active substance in PPP		PPP		Active substance in PPP		PPP		Active substance in PPP	
	Sherpa 100 EC	Dursban 480 EC	Cypermethrin	Chlorpyrifos	Sherpa 100 EC	Mospilan 20 SP	Cypermethrin	Acetamiprid	Karate Zeon 05 CS	Closer	Lambda-cyhalothrin	Sulfoxaflor
	× RAC		ng/µL		× RAC		ng/µL		× RAC		ng/µL	
1*	0	0	0	0	0	0	0	0	0	0	0	0
2	0.25	0	25	0	0.04	0	4	0	0.2	0	5	0
3	0.5	0	50	0	0.2	0	20	0	1	0	25	0
4	1	0	100	0	1	0	100	0	5	0	125	0
5	5	0	500	0	5	0	500	0	25	0	625	0
6	0	0.2	0	192	0	0.04	0	3.2	0	0.2	0	24
7	0.25	0.2	25	192	0.04	0.04	4	3.2	0.2	0.2	5	24
8	0.5	0.2	50	192	0.2	0.04	20	3.2	1	0.2	25	24
9	1	0.2	100	192	1	0.04	100	3.2	5	0.2	125	24
10	5	0.2	500	192	5	0.04	500	3.2	25	0.2	625	24
11	0	0.4	0	384	0	0.2	0	16	0	1	0	120
12	0.25	0.4	25	384	0.04	0.2	4	16	0.2	1	5	120
13	0.5	0.4	50	384	0.2	0.2	20	16	1	1	25	120
14	1	0.4	100	384	1	0.2	100	16	5	1	125	120
15	5	0.4	500	384	5	0.2	500	16	25	1	625	120
16	0	0.7	0	672	0	1	0	80	0	5	0	600
17	0.25	0.7	25	672	0.04	1	4	80	0.2	5	5	600
18	0.5	0.7	50	672	0.2	1	20	80	1	5	25	600
19	1	0.7	100	672	1	1	100	80	5	5	125	600
20	5	0.7	500	672	5	1	500	80	25	5	625	600
21	0	1	0	960	0	5	0	400	0	25	0	3000
22	0.25	1	25	960	0.04	5	4	400	0.2	25	5	3000
23	0.5	1	50	960	0.2	5	20	400	1	25	25	3000
24	1	1	100	960	1	5	100	400	5	25	125	3000
25	5	1	500	960	5	5	500	400	25	25	625	3000
26	Not exposed		0	0	0	0	0	0	0	0	0	0

Table 2. Median lethal time (LT_{50s} ± standard error [SE], days) for each combination of pesticides in the 'Dursban × Sherpa' experiment. Some cells do not have SE because nearly all individuals died within the first 24 hours. Note that the LT_{50s} listed for the control group is the combined LT_{50s} for both the 0.01%Triton X-100 and not exposed bees, as the two groups did not differ.

		Sherpa 100 EC × RAC				
		0	0.25	0.5	1	5
Dursban 480 EC × RAC	0	29±3.2	10±3.1	24±1.6	30±1.7	29±1.3
	0.2	33±0.7	35±1.3	28±3.6	18±4.2	26±3.7
	0.4	20±16.4	18±11.0	19±11.0	29±5.9	24±16.7
	0.7	1	1	1	1	1
	1	1	1	1	1	1

Table 3. Median lethal time (LT_{50s} ± standard error [SE], days) for each combination of pesticides in the 'Mospilan × Sherpa' experiment. Note that the LT_{50s} listed for the control group is the LT_{50s} only for the 0.01%Triton X-100 control, as the survival in two control groups (Triton control and not exposed bees) differed from each other.

		Sherpa 100 EC × RAC				
		0	0.04	0.2	1	5
Mospilan 20 SP × RAC	0	25±8.2	33±3.4	34±2.7	29±3.8	39±2.7
	0.04	40±1.8	37±3.3	31±2.2	40±2.0	36±2.2
	0.2	36±2.7	37±2.3	44±1.1	28±0.6	33±7.4
	1	26±3.4	30±2.7	38±2.3	35±2.7	30±3.3
	5	30±2.7	28±2.2	33±1.8	38±2.7	37±1.1

Table 4. Median lethal time (LT_{50s} ± standard error [SE], days) for each combination of pesticides in the 'Karate × Closer' experiment. Some cells do not have a SE because nearly all individuals died within the first 24 hours. Note that the LT_{50s} listed for the control group is the combined LT_{50s} for both the 0.01%Triton X-100 and not exposed bees, as the two groups did not differ.

		Closer × RAC				
		0	0.2	1	5	25
Karate Zeon 050 CS × RAC	0	19±0.3	21±1.5	14±0.7	1	1
	0.2	16±0.4	13±0.3	14±0.6	1	1
	1	16±0.4	19±0.7	26±0.6	1	1
	5	16±0.4	15±0.9	11±1.0	2±0.9	1
	25	3±.1	2±0.3	3±0.2	1	1

SUPPLEMENTARY MATERIALS

Combined effects of insecticides on survival of the red mason bee *Osmia bicornis*

Anna Misiewicz[1*], Maryellen Zbrozek[2], Ryszard Laskowski[2], Agnieszka J. Bednarska[1]

[1]Institute of Nature Conservation, Polish Academy of Sciences, A. Mickiewicza 33, 31-120 Kraków, Poland

[2]Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

[*]Corresponding author: misiewicz@iop.krakow.pl

TABLES AND FIGURES

Table S1. The concentrations of active substances (a.s.) measured in selected treatments. The chemical analyses were done by the certified external contractor – the Regional Experimental Station of the Institute of Plant Protection, National Research Institute in Białystok, Poland, using LC-MS/MS or GS-MS/MS techniques. The presented values are the means of two measurements.

Experiment	Treatment	Concentration of PPP (×RAC)	Active substance (a.s.) measured	Nominal concentration of a.s. [ng/µL]	Measured concentration of a.s. [ng/µL]
Dursban × Sherpa	Dursban 480 EC	0.4	chlorpyrifos	384	338
	Sherpa 100 EC	0.5	cypermethrin	50	43
	Dursban 480 EC × Sherpa 100 EC	0.4	chlorpyrifos	384	411
		0.5	cypermethrin	50	34
Sherpa × Mospilan	Sherpa 100 EC	0.04	cypermethrin	4	4
		1	cypermethrin	100	208
	Mospilan 20 SP	0.04	acetamiprid	3.2	2
		1	acetamiprid	80	92
	Sherpa 100 EC × Mospilan 20 SP	1	cypermethrin	100	240
		0.04	acetamiprid	80	78
Karate × Closer	Karate Zeon 050 CS	0.2	lambda-cyhalothrin	5	7
		1	lambda-cyhalothrin	25	50
		5	lambda-cyhalothrin	125	252
		25	lambda-cyhalothrin	625	723
	Closer	0.2	sulfoxaflor	24	59
		1	sulfoxaflor	120	196
		5	sulfoxaflor	600	763
		25	sulfoxaflor	3000	2524
	Karate Zeon 050 CS × Closer	0.2	lambda-cyhalothrin	5	8
		0.2	sulfoxaflor	24	61
		5	lambda-cyhalothrin	125	209
		5	sulfoxaflor	600	718

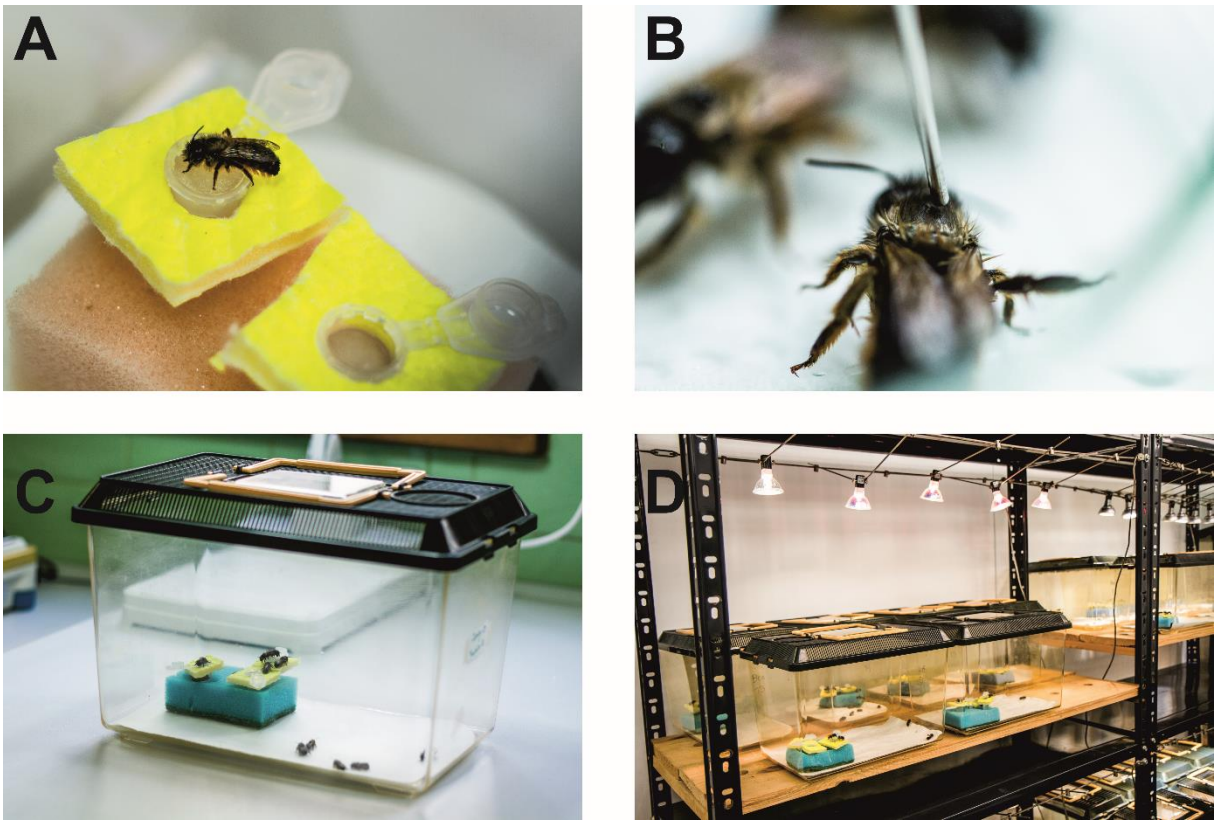


Figure S1. (**A**) Eppendorf tubes used for feeding bees with sucrose solution 33% (w/w), with cotton wool inside to prevent bees from entering the tubes and with a small square-cut piece of yellow sponge-cloth provided around the tube to attract the bees to the food; (**B**) Topical application of the treatment solution to bee female on glass Petri dishes using Hamilton micro-syringe; (**C, D**) Plastic box used for group housing after application of treatment solution.

ARTYKUŁ IV

Misiewicz, A. Filipiak, Z. M., Kadyrova, K., Bednarska, A. J., 2024. Combined effects of three insecticides with different modes of action on biochemical responses of the solitary bee *Osmia bicornis*. Chemosphere, 142233. https://doi.org/10.1016/j.chemosphere.2024.142233 (IF = 8.8; 140 pkt. MNiSW).

Chemosphere 359 (2024) 142233

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Combined effects of three insecticides with different modes of action on biochemical responses of the solitary bee *Osmia bicornis*

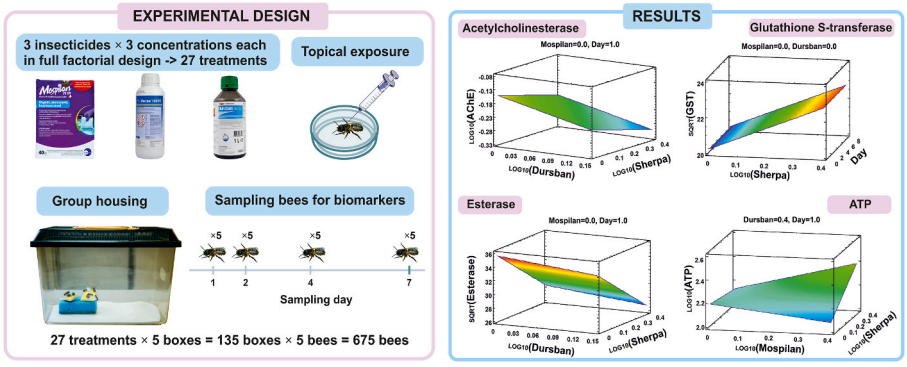
Anna Misiewicz [a,*], Zuzanna M. Filipiak [a], Kamila Kadyrova [b], Agnieszka J. Bednarska [a]

[a] Institute of Nature Conservation, Polish Academy of Sciences, A. Mickiewicza 33, 31-120, Kraków, Poland
[b] Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387, Kraków, Poland

HIGHLIGHTS

- Solitary bees were topically exposed to three insecticides and their mixtures.
- Insecticides affected enzymatic and non-enzymatic biomarkers in bees.
- Dursban 480 EC and Sherpa 100 EC reduced AChE and EST activity.
- Sherpa 100 EC increased GST activity.
- ATP levels varied according to insecticides, their combination and sampling time.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling editor: Patryk Oleszczuk

Keywords:
Mixture toxicity
Acetylcholinesterase
Glutathione S-Transferase
Esterase
ATP
Pollinators

ABSTRACT

Bees are simultaneously exposed to a variety of pesticides, which are often applied in mixtures and can cause lethal and sublethal effects. The combined effects of pesticides, however, are not measured in the current risk assessment schemes. Additionally, the sublethal effects of pesticides on a variety of physiological processes are poorly recognized in bees, especially in non-*Apis* solitary bees. In this study, we used a full-factorial design to examine the main and interactive effects of three insecticide formulations with different modes of action (Mospilan 20 SP, Sherpa 100 EC, and Dursban 480 EC) on bee biochemical processes. We measured acetylcholinesterase (AChE), glutathione S-transferase (GST) and esterase (EST) activities, as well as a nonenzymatic biomarker associated with energy metabolism, i.e., ATP level. All studied endpoints were affected by Sherpa 100 EC, and the activities of AChE and EST as well as ATP levels were affected by Dursban 480 EC. Moreover, complex interactions between all three insecticides affected ATP levels, showing outcomes that cannot be predicted when testing each insecticide separately. The results indicate that even if interactive effects are sometimes difficult to interpret, there is a need to study such interactions if laboratory-generated toxicity data are to be extrapolated to field conditions.

* Corresponding author.
E-mail address: misiewicz@iop.krakow.pl (A. Misiewicz).

https://doi.org/10.1016/j.chemosphere.2024.142233
Received 11 February 2024; Received in revised form 1 May 2024; Accepted 2 May 2024
Available online 3 May 2024

1. Introduction

A growing body of evidence points to a decline in wild pollinating insects, particularly in Europe and North America, where the insect fauna has been widely studied (Koh et al., 2016; Powney et al., 2019), although similar trends are occurring in other regions of the world (Millard et al., 2021). Several factors contribute to the decline of wild pollinators, including the intensification of agriculture and the resulting loss of seminatural habitats (e.g., meadows, field margins, hedgerows) as well as the widespread use of pesticides (Brittain and Potts, 2011; Hallmann et al., 2017). Wild pollinating insects may be exposed to pesticides through various routes, such as oral or contact routes (Sgolastra et al., 2018), and residues of many pesticides have been found not only in the pollen and nectar of crop plants but also in wildflowers growing near crops (Zioga et al., 2020) and soil (Silva et al., 2019). Among the many groups of pesticides used in agriculture, insecticides belonging to neonicotinoids, pyrethroids, and organophosphates are used worldwide to control insect pests. Residues of insecticides are often found in bee pollen (Mullin et al., 2010), and the comprehensive assessment of risks posed by various pesticide groups revealed that the primary risk to honeybees and bumblebees, arising from exposure to contaminated pollen, is associated with residues of the compounds belonging to these three groups (Sanchez-Bayo and Goka, 2014).

Neonicotinoids, pyrethroids, and organophosphates have different modes of action (Sparks and Nauen, 2015). Neonicotinoids act as agonists on the nicotinic acetylcholine receptor, mimicking natural neurotransmitters, disrupting the initiation of electric signals in postsynaptic neurons and causing overstimulation of neuronal activity, which may be lethal (Seifert, 2014). Pyrethroids interfere with voltage-gated sodium channels located in insect neuronal membranes, which ultimately disrupts the transmission of electrical signals in the nervous system. This alteration in the membrane potential of nerve cells induces an abnormal state of hyperexcitability, causing a sublethal “knockdown” effect – paralysis and flight incapability. This state can lead to either death or recovery through enzymatic detoxification (Krief, 2021). Organophosphorus insecticides inactivate acetylcholinesterase (AChE) by an organophosphorus ester: they can bind to AChE and block the breakdown of acetylcholine (ACh). This leads to excessive release of ACh followed by overstimulation of nicotinic receptors (Fukuto, 1990). Notwithstanding these differences, all three classes of insecticides aim to disrupt normal neural function in insects, leading to their incapacitation or death. To protect honeybees against the toxic effects of these insecticides, the European Union has banned three neonicotinoids (thiamethoxam, imidacloprid, and clothianidin) (OJEU, 2018a, 2018b, 2018c), three pyrethroids (alpha-cypermethrin, beta-cypermethrin, beta-cyfluthrin) (OJEU, 2017, 2020a, 2021), and a few organophosphates, including chlorpyrifos (OJEU, 2020b) and phosmet (OJEU, 2022). However, those and many other insecticides are used worldwide in agricultural landscapes (757,540 tones of insecticides in 2021; Food and Agriculture Organization of the United Nations, 2021), with the risk of lethal or sublethal effects on bees and other insect pollinators (Onwona-Kwakye et al., 2020).

The individual effects of different neonicotinoids, pyrethroids, and organophosphates on bees are well documented, but the combined effects of these insecticides in their binary or ternary mixtures on bees, especially wild bees, are poorly recognized (Tosi et al., 2022) and most of the binary combinations effects that can occur in the field are unknown (Barascou et al., 2019; Carnesecchi et al., 2019; Siviter et al., 2021). This is probably at least partly because the current ecological risk assessment does not consider the impact of interactions between pesticides (EFSA et al., 2023). However, under field-realistic conditions, the usual agricultural practice is to mix several pesticides, often from different groups, or apply them at short intervals to control a wider range of pests or increase the effectiveness of the mixture (Heys et al., 2016). The use of insecticide mixtures has been proposed as a strategy to limit the emergence of pest resistance, although not without an impact on pollinating insects (Taillebois and Thany, 2022). In an agricultural landscape, bees are also exposed to multiple pesticides because they forage on different crops (e.g., oilseed rape, fruits, vegetables, cotton) sprayed with different pesticides, and exposure through noncrop plants (e.g., weeds, wildflowers, succeeding crops) has also been reported (McArt et al., 2017). As shown by several studies, many pesticides in mixtures can show synergistic effects, meaning that the combined effect is stronger than a simple summation of individual effects, resulting in increased mortality (Siviter et al., 2021). Pesticides can also exhibit antagonistic relationships (when the combined effect is weaker than the simple summation), resulting in reduced mortality (Ritz et al., 2021), but such effects have been reported less frequently (Carnesecchi et al., 2019). The interactive effects of pesticides can vary depending on the chemical structure of the compound, dosage levels, biological targets, and duration of exposure (Hernández et al., 2017). Based on the dose–response acute contact data for 92 binary mixtures available for honeybee (*Apis mellifera*) and wild bees (*Bombus* spp., *Osmia* spp.), dose addition, synergism and antagonism were found in 17%, 72% and 11% of cases, respectively (Carnesecchi et al., 2019). Additionally, a review published recently by Taillebois and Thany (2022) indicated that neonicotinoid and pyrethroid mixtures as well as neonicotinoid and organophosphate mixtures may result in diverse toxicological effects (i.e., antagonism, additivity, or synergism) in different nontarget insects, including bees. Apart from the effects of individual active substances, coformulants used in plant protection products (PPP) can also affect bees (Heys et al., 2016). In the field, formulations containing less than 50% of the active substances are used, with the remaining components comprising solvents, activators, spreaders, stickers, adjuvants, and surfactants, which act as enhancers of the active substances in the product (Mullin et al., 2015). These substances are frequently used as "other ingredients" in pesticide labels (EPA, 2023). Studies showed that they could be toxic to both larval and adult honeybees (Shannon et al., 2023; Zhu et al., 2014). For this reason, it is important to study not only the effect of mixtures of different active substances but also the effects of mixtures of PPPs on nontarget insects.

Historically, the assessment of potential pesticide effects on pollinators has mainly focused on honeybees (*A. mellifera*). However, there is a growing need to encompass a wider range of bee species with different biology and ecology (Schmolke et al., 2021). Solitary bees, such as *Osmia* spp. have been recognized as valuable model species when exploring pesticide risk to bees and thus for ecological risk assessments (EFSA et al., 2023). The inclusion of *Osmia* spp. as model species in the evaluation of pesticide effects on pollination can be justified by several factors, such as their role as pollinators for various crops (i.e., apples, cherries, oilseed rape) (Bosch and Kemp, 2002) and the potential differences in pesticide sensitivity when compared to honeybees and bumblebees (Heard et al., 2017). However, data on the combined effects of insecticides on *Osmia* spp. are scarce, particularly concerning sublethal effects assessed at the biochemical level (Lehmann and Camp, 2021). A better understanding of the risks associated with the combined use of insecticides and their effects on the physiology and metabolism of these important pollinators is a crucial step for their better protection (Leroy et al., 2023).

Different physiological sublethal effects of pesticides have already been reported in honeybee (Decourtye et al., 2005) and non-*Apis* bees (Martins et al., 2023), including effects on AChE and glutathione S-transferase (GST) (Chibee et al., 2021), the latter having a central role in detoxification as an important mediator of oxidative stress responses (Ranson and Hemingway, 2005). While AChE remains a widely used biomarker within the class of esterases, the activity of esterases as a group of enzymes has also been adopted as an indicator of pesticide exposure in insects (Bosch-Serra et al., 2021), including bees (Ahmed et al., 2023; Milone et al., 2020; Zhu et al., 2017a, 2017b). Esterase (EST) enzymes are categorized based on their function and substrate specificity, and play broad and pivotal functions in metabolism (Wei et al., 2020). They improve the detoxification process of insecticides by

breaking down ester bonds and converting them into less harmful (water-soluble) forms that can be more easily eliminated from the body (Montella et al., 2012). Moreover, adenosine triphosphate (ATP) is gaining recognition for its potential to assess pesticide effects in insects (Kairo et al., 2017). ATP plays a vital role in transferring and storing energy within living organisms (Dunn and Grider, 2023), supporting essential cellular processes and maintaining metabolic functions (Kairo et al., 2017). ATP also serves as an oxidative stress indicator, wherein increased oxidative stress leads to amplified production of H_2O_2 by mitochondrial electron transport, depletion of ATP, and ultimately, cell death (Tiwari et al., 2002). Nevertheless, knowledge of the effects of insecticides on ATP in bees is limited to studies on honeybees and bumblebees (Kairo et al., 2016, 2017; Powner et al., 2016; Prado et al., 2020).

In this study, we investigated the possible interactive effects of three commercially available PPPs: neonicotinoid Mospilan 20 SP (active substance (a.s.) acetamiprid), pyrethroid Sherpa 100 EC (a.s. cypermethrin), and organophosphate Dursban 480 EC (a.s. chlorpyrifos), applied at their realistic field concentrations, on the activity of three enzymes (AChE, EST, GST) and the whole-body ATP level in the red mason bee *O. bicornis*. Since the optimal time to measure biomarker response depends on the type of pesticide, dose, route of exposure, and the organism under study (Ahmed et al., 2023; Bednarska et al., 2017; Han et al., 2019), the levels of biomarkers were measured at four time points after topical application of insecticides and their mixtures.

2. Materials and methods

2.1. Study organism

Osmia bicornis cocoons were purchased from a local supplier (BioDar, Poland) in February 2021 and kept at a wintering temperature of 4 °C until use. In April, the largest cocoons, expected to be females, were placed in plexiglass boxes (46 × 30 × 17 cm) with airflow provision from the top (Fig. S1A) and incubated at 20 ± 2 °C, 60 ± 5% relative humidity (RH) and the 16:8 h light:dark (L:D) regime until emergence. Boxes were controlled several times a day, and the newly emerged males were released, while females were fed *ad libitum* with sucrose solution 33% (w/w) placed in 2-mL Eppendorf tubes with a cotton wool stopper and small square-cut yellow sponge cloth provided around the tube (Fig. S1B). At least 4-day-old females were used to avoid a cohort effect on the duration of bee survival (Robinson et al., 2017).

2.2. Insecticides

Three PPPs available on the market were used: Mospilan 20 SP (Mospilan) with 20% acetamiprid a.s. (Sumi Agro, Warszawa, Polska), Sherpa 100 EC (Sherpa) with 10.76% cypermethrin a.s. (Chemirol, Mogilno, Poland), and Dursban 480 EC (Dursban) with 44.86% chlorpyrifos a.s. (Dow AgroSciences, Warszawa, Poland). Stock solutions of each PPP were prepared in 100 mL of 0.01% Triton X-100 (used to facilitate the adhesion of the solution to the bee body) as 10 × Recommended Application Concentration (RAC) based on the recommended field application rates given by their manufacturers for spray application in oilseed rape crop and the recommended dilution (300 L/ha). Then, stock solutions were diluted in 0.01% Triton to achieve the desired range of concentrations of individual insecticides and their mixtures (Table S1). The following concentrations, expressed as a fraction of RAC, were used: 0, 0.5 and 1 × RAC for Mospilan and Sherpa and 0, 0.2 and 0.4 × RAC for Dursban due to its higher toxicity to *O. bicornis* (Mokkapati et al., 2021). Mokkapati et al. (2021) showed that in topical exposure, the estimated infinity LC_{50} value ($LC_{50\infty}$) for Dursban was approximately 70% lower than the concentration recommended for field application for this product, whereas for Sherpa and Mospilan, it was much higher than RAC. Also, our earlier study confirmed high toxicity of Dursban (0.7 × RAC caused 100% mortality within 24 h after topical exposure; *data not shown*). Therefore, for this study, we chose lower than recommended concentrations of Dursban (0, 0.2 and 0.4 × RAC) to ensure that enough bees would survive the study and be collected for biomarker measurements for at least a week.

2.3. Experimental design

Two identical experiments were conducted in a short time interval (ca. two weeks apart). In the first experiment, bees were sampled for enzyme activity measurements, and in the second experiment, ATP levels were measured. The full factorial design was used with three levels of each PPP, which resulted in 27 treatments, 5 boxes per treatment, and a minimum of 5 bees in each box (i.e., min. 25 bees per treatment in each experiment) but more bees than needed for the analyses were used in treatments with higher insecticide concentrations to account for elevated mortality (Table S2).

Approximately 1 h before each experiment, female bees taken from the breeding boxes were placed in glass Petri dishes (5 bees/dish) and transferred to a refrigerator (4 °C) for approximately 20 min to limit their mobility and ensure proper pesticide application (i.e., prevent the bees from spreading the solution to the neck or wing hinges). Bees were treated individually by topical application of 1 μL of the test solution (either insecticide(s) solution or 0.01% Triton X-100 solution as a solvent control) on the dorsal thorax using a 50 μL Hamilton microsyringe with a repeater (Fig. S1C). Such exposure simulates direct spraying of foraging bees in crops with high bee attractiveness. The exposed bees were then transferred to plastic boxes (min. 5 bees per box, five boxes per treatment; Fig. S1D) and moved to the climatic chamber (20 ± 2 °C, 60 ± 5% RH, 16:8 L:D). Throughout the experiment, the bees were provided food *ad libitum* with 33% (w/w) sucrose solution in Eppendorf tubes (as described above; Fig. S1B) and observed daily. On each sampling day (i.e., 1, 2, 4 and 7 days after the exposure), the bees were sampled (one bee per box, five bees per treatment), frozen in liquid nitrogen and stored at −80 °C for analyses.

2.4. Enzyme activity and protein analysis

Before homogenizing the bees for the analyses, the most heavily sclerotized parts of their exoskeleton (legs and wings) were removed (on ice). The bees were homogenized individually on ice in 500 μL of phosphate buffer (50 mM KH_2PO_4, 40 mM K_2HPO_4 and 0.1% (w/v) Triton X-100, pH 7.4) using a homogenizer (Bead Ruptor Elite, Omni International). Next, the homogenates were centrifuged for 10 min at 4 °C and 15,000 *g* (Eppendorf Centrifuge 5430 R), and the resulting supernatants were stored at −80 °C for the analyses.

The acetylcholinesterase activity was assayed according to the modified method by Bednarska et al. (2017). The reaction mixture contained 5 μL of supernatant, 180 μL of phosphate buffer (pH 7.4), and 10 μL of 0.01 M DTNB (5.5′-dithiobis [2-nitro-benzoic acid], Sigma–Aldrich, USA) in 0.1 M Tris-HCl (pH = 8.0; BioChemika) and 5 μL of 0.1 M of acetylthiocholine iodide ((2-mercaptoethyl) trimethylammonium iodide acetate; Sigma–Aldrich, USA)) as a substrate. AChE activity was determined spectrometrically as kinetic readings of absorbance in 42 s intervals over 4 min at λ = 405 nm and then expressed as nmol hydrolysed acetylthiocholine iodide per min per mg protein (nM/min/mg protein).

The activity of GST was determined using a GST assay kit (CS0410, Sigma–Aldrich, USA) according to the manufacturer's protocol. The reaction mixtures consisted of 5 μL supernatant and 195 μL Master mix (0.1 mL 200 mM L-Glutathione reduced, 0.1 mL 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) (substrate) and 9.8 mL Dulbecco's Phosphate Buffered Saline). The absorbance was recorded spectrophotometrically in 60 s intervals over 6 min at λ = 340 nm, and GST activity was expressed as nmol CDNB conjugate formed per min per mg protein (nM/min/mg protein).

The EST activity was measured according to a protocol prepared

based on Bosch-Serra et al. (2021), Johnston and Ashford (1980) and Milone et al. (2020). The reaction started by adding 20 μL of supernatant to 160 μL of distilled water and 20 μL of substrate solution 1 mM 1-NA (1-naphthyl acetate; Sigma–Aldrich, USA) (diluted in distilled water with 1% acetone (≥99.5%)). The mixture was incubated at 30 °C for 10 min, and the enzymatic reaction was terminated by adding 50 μL of staining solution (0.075 g Fast Blue B salt (Pol-Aura, Poland) dissolved in 16.25 mL distilled water and 8.75 mL of 10% sodium dodecyl sulfate solution (Sigma–Aldrich, USA)). The product of the new reaction between 1-NA and the Fast Blue B salt was determined spectrophotometrically by measuring the absorbance in 60 s intervals over 5 min at $\lambda = 570$ nm. To obtain a standard curve, 1-NA was replaced by α-naphthol (1-naphthol solution 20% in ethanol; Chempur, Poland), and the supernatant was replaced by homogenization phosphate buffer (pH = 7.4). Seven concentrations (0, 0.063, 0.125, 0.25, 0.5, 1 and 2 mM/mL) of α-naphthol in ethanol (96%) were prepared to calibrate the absorbance versus concentration of α-naphthol. The EST activity was expressed as nmol of hydrolysed 1-NA per min per mg protein (nM/min/mg protein).

The protein concentrations were determined according to the method of Bradford (1976) by using Bradford's reagent (Sigma–Aldrich, USA) at 1:50 and measuring the absorbance at $\lambda = 595$ nm using bovine serum albumin (Sigma–Aldrich, USA) as a standard.

All absorbance measurements were carried out at room temperature (25 °C) on 96-well plates (Greiner Bio-One, GmbH, Austria; MSCPNUV40, Millipore, Sigma–Aldrich, USA) using a BioTek Synergy HTX multimode reader (Agilent Technologies, USA). Each sample was measured in three replicates on a plate, and the average absorbance corrected against blank sample (i.e., the sample consisting of the homogenization buffer (phosphate buffer) instead of supernatant and all components of the reaction mixture) was used for further calculations.

2.5. ATP level analysis

For ATP measurements, the frozen bees were first weighed on ice to the nearest 0.1 mg (WPA-180/K Radwag, Poland) and immediately afterwards individually homogenized on ice in 500 μL of 1 M perchloric acid (Sigma–Aldrich, USA) using a homogenizer (Bead Ruptor Elite, Omni International). Next, the samples were placed on ice for 12 min, after which they were vortexed and centrifuged (10 min, 4 °C, 12,000 *g* (Eppendorf Centrifuge 5430 R)). The supernatants were neutralized with a mixture of 2 M KOH (Avantor Performance, Poland) and Tris hydrochloride solution (100 mM, pH = 7.8, Sigma–Aldrich, USA) (Nicodemo et al., 2020). After final centrifugation (5 min, 4 °C, 8000 *g*), the samples were immediately used for ATP ratio luminometric measurements using an ATPlite Luminescence ATP Detection Assay System (PerkinElmer, USA) according to the manufacturer's protocol. Measurements were performed on white 96-well plates (OptiPlate-96, PerkinElmer, USA) using a BioTek Synergy HTX multimode reader (Agilent Technologies, USA). Each sample was measured in three replicates, and the average ATP levels were expressed as nmol of ATP per mg of body mass (nM/mg body mass).

2.6. Data analysis

To quantify the relationship between the measured endpoints (enzyme activities and ATP level) and the studied factors (Mospilan, Sherpa, Dursban, and sampling day), the data were analysed with general linear models (GLMs). The log-transformed insecticide concentrations ($\log_{10}$(concentration+1)) were used as independent variables. The analysis started with formulating the full model, i.e., testing all main factors and interactions for significance ($p \leq 0.05$), and then a backwards selection with retained lower order effect was used to remove from the model nonsignificant factors (and/or their interaction(s)). The normal distribution of residuals was formally tested using the Shapiro–Wilk W test. Since the normal distribution of residuals was not met, data for AChE activity and ATP level were log-transformed, while data for GST and EST activity underwent square root transformation (Zar, 1999). All data analyses were performed using Statgraphics Centurion 19 (Statgraphics Technologies Inc., version 19.4.04).

3. Results

Due to some random mortality (Table S2), data for 534 bees, instead of the planned 540 (27 treatments × 5 bees × 4 sampling days), were used for statistical analysis of AChE activity, and 532 bees were used for GST and EST activity measurements, as there was not enough homogenate in two samples to run the measurements (Table S1). In the case of ATP, data for 400 bees sampled on Days 1, 2, and 4 were used in the statistical analysis due to the mortality of bees on Day 7, especially in treatments with high concentrations of PPPs, especially with high concentration of Dursban (Tables S1 and S2). The mean values for all biomarkers (AChE, GST, EST activity [nM/min/mg protein] and ATP level [nM/mg body mass] in female *Osmia bicornis* bees are presented in Tables S3–S6.

The final GLMs, i.e., after a backwards selection with retained lower order effect, for all enzymes were significant at $p \leq 0.0001$, with no significant effect of Mospilan and no significant interactions between the insecticides tested (Table 1). Dursban and Sherpa significantly decreased AChE activity ($p \leq 0.0001$ and $p = 0.03$, respectively; Fig. 1A), but the effect of Sherpa depended on the sampling day ($p = 0.04$ for Sherpa × sampling day interaction), with a steeper relationship on later days. The sampling day itself was also significant ($p = 0.002$), but due to the significant interaction with Sherpa, AChE activity increased with time (without Sherpa or at low Sherpa concentrations), remained constant (at moderate Sherpa concentrations) or increased (at higher Sherpa concentrations) (Fig. 1B). The activity of GST significantly increased with increasing Sherpa concentration ($p \leq 0.0001$; Fig. 1C), and this was the only factor affecting this enzyme. Both Dursban ($p = 0.008$) and Sherpa ($p \leq 0.0001$) decreased the EST activity with increasing concentration, with no differences between sampling

Table 1
The results of GLM analysis for biomarkers: acetylcholinesterase (AChE), glutathione S-transferase (GST), esterase (EST) activity [nM/min/mg protein] and ATP level [mM/mg body mass] measured at different sampling days after exposing the red mason bee *Osmia bicornis* to Mospilan 20 SP (Mospilan), Sherpa 100 EC (Sherpa), and Dursban 480 EC (Dursban) in a full-factorial experiment with three concentrations of each insecticide. The *p* values for the variables and/or interactions included in the final model, i.e., the model with only significant explanatory variables and/or interactions (at $p \leq 0.05$) retained after backwards stepwise selection while holding lower order effects, are presented together with *p* values and R^2 for the final model.

Factor/Interaction	Biomarker measured			
	AChE	GST	EST	ATP
Mospilan				0.03
Sherpa	0.03	≤0.0001	≤0.0001	≤0.0001
Dursban	≤0.0001		0.008	0.004
sampling day	0.002			0.04
Mospilan × Sherpa				0.007
Mospilan × Dursban				0.02
Sherpa × Dursban				≤0.0001
Mospilan × sampling day				
Sherpa × sampling day	0.04			0.001
Dursban × sampling day				
Mospilan × Sherpa × Dursban				0.001
Mospilan × Sherpa × sampling day				
Mospilan × Dursban × sampling day				
Sherpa × Dursban × sampling day				0.03
Mospilan × Sherpa × Dursban × sampling day				
p for the final model	≤0.0001	≤0.0001	≤0.0001	≤0.0001
R^2 [%]	18.7	7.9	17.0	12.9

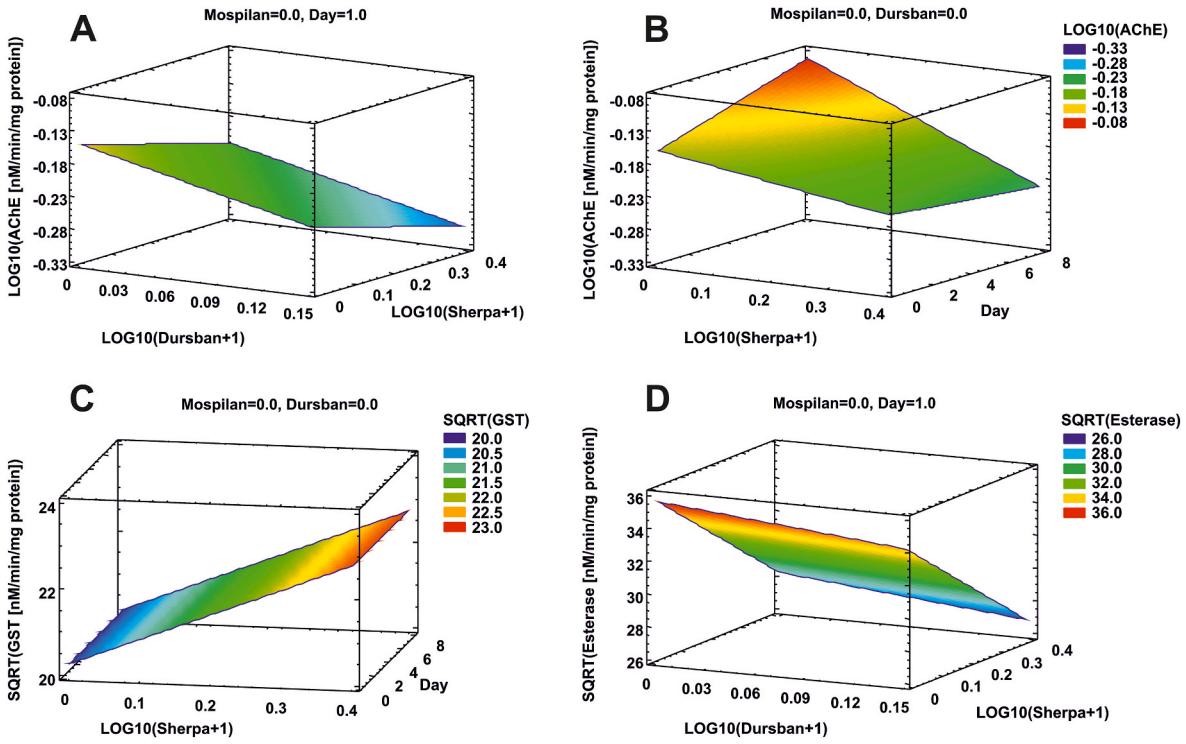


Fig. 1. Estimated response surfaces for (A) effect of Dursban 480 EC (Dursban) and Sherpa 100 EC (Sherpa) (plotted for no Mospilan 20 SP (Mospilan) at sampling Day 1) and (B) effect of the interaction between Sherpa and sampling day (day) (plotted for no Dursban and no Mospilan) on acetylcholinesterase (AChE) activity [nM/min/mg protein], as well as (C) effect of Sherpa (plotted for different sampling days and in the absence of other insecticides) on glutathione S-transferase (GST) activity [nM/min/mg protein], and (D) effect of Dursban and Sherpa (plotted for no Mospilan and sampling Day 1) on esterase (EST) activity [nM/min/mg protein], in full factorial experiment with female *Osmia bicornis* exposed topically to insecticide(s).

days (Fig. 1D).

The final model for ATP, including all significant variables and interactions, was significant at $p \leq 0.0001$ and explained 13% of the variance (Table 1). The analysis revealed significant effects on the ATP level of all insecticides (Mospilan $p = 0.03$, Sherpa $p \leq 0.0001$, Dursban $p = 0.004$) and sampling day ($p = 0.04$)) and several interactions between these factors (Table 1). An increase in ATP levels on the first day after exposure to Sherpa was followed by a decrease on subsequent

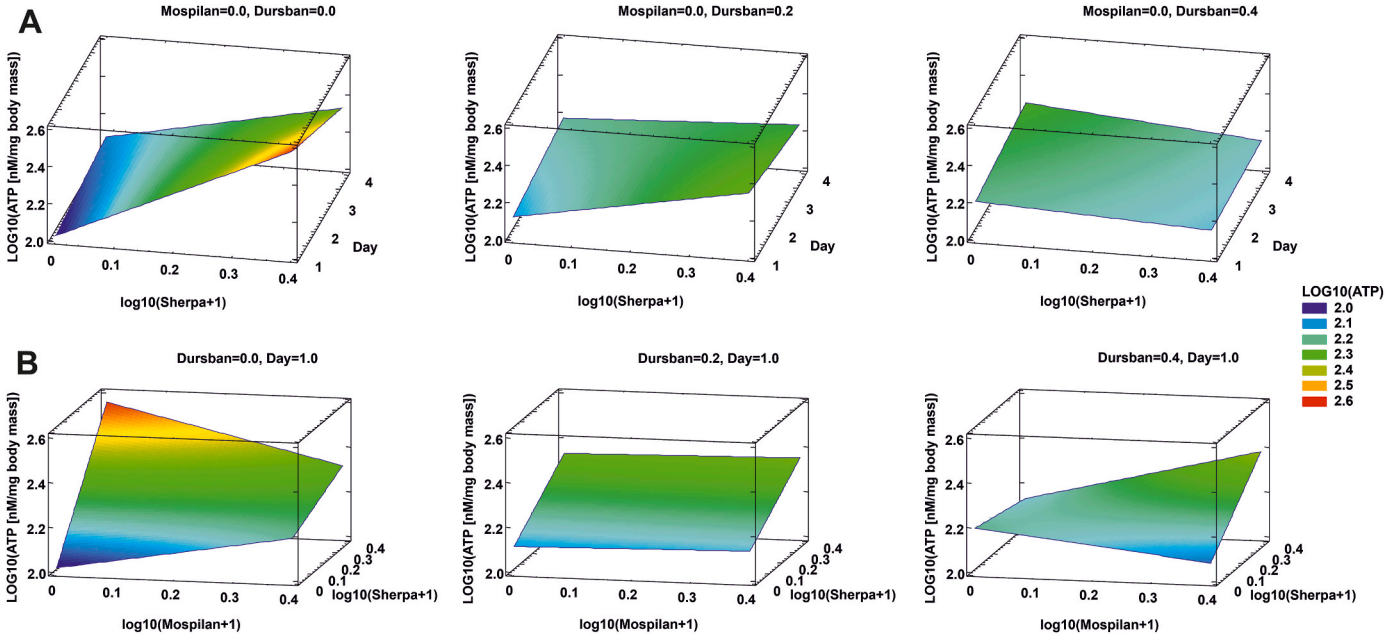


Fig. 2. Estimated response surfaces for (A) effect of interaction between Sherpa 100 EC (Sherpa) and sampling day (day) (plotted for different concentrations of Dursban 480 EC (Dursban) in the absence of Mospilan 20 SP (Mospilan) exposure) and (B) effect of interaction between Mospilan and Sherpa (plotted for different concentrations of Dursban and sampling Day 1) on the ATP level [nM/mg body mass] in full factorial experiment with female *Osmia bicornis* exposed topically to insecticide(s).

sampling days, as shown by a significant interaction between Sherpa and sampling day ($p = 0.001$; Fig. 2A). Dursban reduced the positive impact of Sherpa on ATP levels on the first day ($p \leq 0.0001$ for Sherpa × Dursban interaction), and the effect decreased on the second day and disappeared on the fourth sampling day, as indicated by the Sherpa × Dursban × sampling day interaction ($p = 0.03$; Fig. 2A). The analysis revealed that Dursban also reduced the impact of Mospilan on ATP levels (significant interaction at $p = 0.02$; Fig. 2B). It was also observed that Mospilan counteracted the effects of Sherpa on ATP levels ($p = 0.007$ for the Mospilan × Sherpa interaction; Fig. 2B). Additionally, a significant interaction between three insecticides was observed ($p = 0.001$): adding Dursban to the insecticide mixture caused the antagonistic effect between Mospilan and Sherpa to disappear (Fig. 2B).

4. Discussion

This study is among a limited number of investigations examining the effects of insecticides on the physiology of the solitary bee (Martins et al., 2023; Mokkapati et al., 2022), and to our knowledge, no previous studies have investigated the interactive effects of insecticides with different modes of action at environmentally realistic concentrations on *O. bicornis*. We found inhibitory effects of Dursban and Sherpa on the activity of both AChE and EST, and exposure to Sherpa led to a significant increase in GST activity. No interactive effects were found between the studied insecticides at environmentally relevant concentrations (i.e., not higher than recommended for field application) for any of the studied enzymes, but both two- and three-insecticide interactions were found in effects on the ATP level. In general, the ATP level exhibited a complex pattern of response to insecticide exposure, including higher-level interactions with time, as in the case of the antagonistic effect of Dursban on Sherpa-induced ATP changes being dependent on the sampling day. AChE inhibition is particularly important because suppression of the activity of this enzyme alters the motor performance of bees (Williamson et al., 2013), which may affect foraging activity and put bees at risk. GST activity increased with Sherpa concentration, which may reflect a defense and detoxification mechanism in bees against pyrethroids that would contribute to the resistance to this class of insecticides, as observed in other insects (Kostaropoulos et al., 2001). Although temporarily beneficial, resistance may be metabolically costly (Sibly and Calow, 1989) and indeed the results for ATP indicate increased metabolic demand for detoxification processes upon exposure to each insecticide alone.

The observed changes in the studied biomarkers show that topical exposure to insecticides resulted in sublethal effects in *O. bicornis*, which may ultimately affect their overall health and survival. Of course, in nature it is unlikely that each bee will be exposed to exactly 1 μL of insecticide solution, so this is rather worst-case scenario. On the other hand, in nature bees are often exposed not only while flying, but also while consuming food resources (nectar or/and pollen), collecting water and soil to build their nests, etc. In addition, in the wild, bees are often exposed to other factors (biotic and abiotic) which can also affect the biomarkers studied. We studied the effect of field realistic doses of insecticides under fully controlled laboratory conditions, thus eliminating stressors other than insecticides. This allowed us to infer potential toxic effects of studied insecticides and their mixture on bee physiology rather than effects on bee populations in the wild. Nevertheless, the biomarkers studied may serve as a good tool to increase the precision of environmental monitoring for solitary bees and provide an early warning system for exposure to different insecticides, as changes in their levels were evident, even without serious effect on bee behaviour.

4.1. Effect of insecticides on AChE activity

As expected, the activity of AChE in *O. bicornis* females decreased in Dursban-exposed bees, which is consistent with studies on *A. mellifera* exposed to the organophosphate insecticide Bracket 97 (acephate a.s.) either continuously via food (Yao et al., 2018) or via single spray (Zhu et al., 2017b). On the other hand, Mokkapati et al. (2022) did not find any changes in AChE activity in *O. bicornis* orally exposed to Dursban 480 EC, Sherpa 100 EC or Mospilan 20 SP. However, the concentrations used in that study in sucrose solution offered to the bees as food were very low (0.0001 × RAC for Dursban 480 EC, i.e., 0.1 μg/mL for chlorpyrifos, 0.2 × RAC for Sherpa 100 EC, i.e., 20 μg/mL for cypermethrin and 0.1 × RAC for Mospilan 20 SP, i.e., 8 μg/mL for acetamiprid), as the study was designed to simulate exposure via nectar. In our study, we used topical exposure to simulate the exposure via spray application, but because of the high toxicity of Dursban 480 EC to *O. bicornis* (Mokkapati et al., 2021), we still used Dursban 480 EC concentrations much lower than the RAC (0.2 and 0.4 × RAC, i.e., 192 and 384 μg/mL, respectively). Recommended application concentrations and corresponding to 0.5 RAC was used for both Sherpa (100 μg/mL and 50 μg/mL, respectively) and Mospilan (80 μg/mL and 50 μg/mL, respectively).

Although pyrethroids are primarily known to affect the voltage-gated Na^+ channels of nerve cell membranes, there are studies suggesting that they can also have secondary effects on the cholinergic system, causing a decrease in AChE activity, which contributes to their neurotoxicity (Kumar et al., 2009). The decrease in AChE activity may be because pyrethroids reduce the release of ACh by increasing the release of gamma-aminobutyric acid (GABA), which leads to the inhibition of cholinergic excitation; at high doses, pyrethroids may affect the sodium channels of GABAergic nerves, leading to an increase in GABA release, which can inhibit the release of ACh from cholinergic nerve terminals (Hossain et al., 2004). Indeed, we found a decrease in AChE activity with increasing Sherpa concentration. This relationship was true for all sampling days, but because in the absence of Sherpa and at low concentrations, AChE activity increased with time, whereas at higher Sherpa concentrations, it remained constant or even slightly decreased, the strength of that negative relationship between AChE activity and Sherpa concentration increased on consecutive bee sampling days. Similarly, Badiou et al. (2008) revealed a decrease in AChE activity in honeybees exposed topically to deltamethrin at 25 ng/bee one day after exposure, but two days after exposure, an increase in AChE activity was observed at this dose. At the same time, no changes in AChE activity compared to the control were observed in honeybees exposed to a lower dose, 12.5 ng/bee (Badiou et al., 2008). It should be noted, however, that most of the honeybees were paralyzed within the first day in the experiment performed by Badiou et al. (2008), and AChE activity was measured in those paralyzed bees 2 days later, which might affect the results. In our study, we selected for biomarker analysis only those bees that were not visibly affected by the tested insecticides in a way that altered their behaviour, i.e., only those without visible signs of stress or disease were sampled. However, since mortality was evident in some treatments, it also means that the most resistant bees that survived were sampled. Our findings also differ from those obtained by Chibee et al. (2021) for *Meliponula bocandei* bees exposed to sublethal doses of cypermethrin (25, 50, 100, 200 and 400 mg/L) via 4-h contact with pesticide-infused filter paper, in which a dose-dependent increase in AChE activity was found. The differences observed between our study and the findings of Badiou et al. (2008) and Chibee et al. (2021) could be attributed to the usage of pesticide formulations in our study compared to the use of only active substances in their studies.

We did not observe any changes in AChE activity under the influence of the neonicotinoid Mospilan. Similarly, Han et al. (2019) reported that acetamiprid at 3.66 μg/mL and 9.15 μg/mL in food did not affect AChE activity in orally exposed newly emerged and forager *Apis c. cerana* bees, regardless of exposure time (1, 5, and 10 days). However, Badawy et al. (2015) found a decrease in AChE activity with increasing Mospilan concentration (0.6–60 μg/mL for acetamiprid in sucrose solution) in *A. mellifera* upon 24 h of continuous oral exposure.

4.2. Effect of insecticides on glutathione S-transferase activity

Sherpa, but not two other insecticides or their combinations, affected GST activity in *O. bicornis*. GST contributes to cellular protection against oxidative damage, and therefore, the induction of GST activity has been used as an environmental biomarker of exposure to toxicants (Badiou-Bénéteau et al., 2012). Indeed, increased GST activity with increasing concentration of Sherpa was found in our study. In contrast, Mokkapati et al. (2022) did not observe any changes in GST activity in the same species, but in their experiment, the bees were orally exposed to 0.2 × RAC of Sherpa (i.e., 20 μg/mL of cypermethrin). Moreover, Zhu et al. (2017b) found significantly decreased GST activity in *A. mellifera* two days after exposure (via spray tower) to the pyrethroid insecticide, but in that study, Karate Zeon 50 CS with λ-cyhalothrin as an active substance was used, which might be responsible for the difference in the GST reaction. Similar to our study, no effect of organophosphate insecticides, either Bracket 97 (Zhu et al., 2017b) or ethion (Delkash-Roudsari et al., 2022), on GST activity was found in orally exposed *A. mellifera*, although given the involvement of organophosphate pesticides in glutathione-dependent metabolism of insecticides and the conjugation of glutathione to organophosphates (Enayati et al., 2005), an increase in GST activity was expected. Such an increase in GST activity relative to untreated bees was found in *A. mellifera* topically exposed to chlorpyrifos (Fellows et al., 2022).

In our study, similar to that by Mokkapati et al. (2022), the neonicotinoid Mospilan did not affect GST activity in *O. bicornis*. In turn, in honeybees exposed to three doses of thiamethoxam, namely, LD_{50} = 51.16 ng/bee and two sublethal doses of 5.12 ng/bee and 2.56 ng/bee, the two higher doses also did not affect GST activity, but increased GST activity was found at the lowest dose two days after exposure (Badiou-Bénéteau et al., 2012). No GST activity changes were found in newly emerged *A. c. cerana* bees following one-day oral exposure to acetamiprid at 3.66 μg/mL (Han et al., 2019), but after 10 days of exposure (every day, the bees had access to contaminated food for 10 h per day and during the remaining 14 h were offered uncontaminated food), a notable GST decrease was noted compared to controls. Conversely, forager bees exposed orally to acetamiprid at 9.15 μg/mL exhibited increased GST activity after one day of exposure in comparison with the control, with no differences after five days of exposure (Han et al., 2019). Therefore, it seems that induction, inhibition and/or inactivation of GST may depend on the physiological differences between life stages, species, exposure doses, time of exposure and sampling time after exposure.

4.3. Effect of insecticides on esterase activity

Only the organophosphate Dursban and pyrethroid Sherpa affected EST, decreasing its activity. Similarly, Stuchi et al. (2023) observed a decrease in EST activity after one day of contact exposure with filter paper soaked with different solutions of the organophosphate insecticide Malathion 500 EC (i.e., 0.031 μg/mL and 0.037 μg/mL malathion a.s.) in the eusocial stingless bee *Tetragonisca. angustula*. Yao et al. (2018) also reported significant suppression of EST activity in *A. mellifera*, but in a different experimental setup, i.e., with bees orally exposed to Lorsban 500 EC (i.e., 0.83 μg/mL of chlorpyrifos a.s.) for 3 weeks. On the other hand, topical exposure of honeybees (*A. mellifera*) to 1 μl of chlorpyrifos at 37.5 μg/mL (i.e., 37.5 ng/bee in comparison with 19.2 ng/bee and 38.4 ng/bee in our study) did not alter general esterase activity relative to control bees (Fellows et al., 2022). Nevertheless, although honeybees (Fellows et al., 2022) and solitary bees (our study) were exposed to similar concentrations of chlorpyrifos, the solitary bees received a higher dose per mg body mass, as they are much smaller than honeybees. The difference in response in these two studies may also result from the fact that we used a formulation (Dursban 480 EC) with chlorpyrifos as an active substance, while Fellows et al. (2022) used the active substance alone.

Detoxification of pyrethroids can be accomplished through the hydrolysis of ester bonds within pyrethroid molecules (Montella et al., 2012), potentially resulting in increased EST activity to neutralize the toxin more effectively. Indeed, increased EST activity in response to pyrethroids was found in various insect species, although mostly for insecticide-resistant strains or populations (Bhatt et al., 2020). To date, literature addressing EST activity in pyrethroid-exposed bees remains limited. Nonetheless, Carvalho et al. (2013) examined three forms of carboxylesterases (CaEs) classified by substrate specificity for hydrolysis: CaE-1 (α-NA), CaE-2 (β-NA), and CaE-3 (ρ-NPA) in tissues of *A. mellifera* after topical exposure to 1 μL of deltamethrin solution (0.05 LD_{50} and 0.1 LD_{50}) and found that α-NA decreased at the highest dose of insecticide, β-NA increased regardless of the dose, and there was no significant change in the activity of ρ-NPA. The results for α-NA in the study by Carvalho et al. (2013) are consistent with our results for the esterase (we also used α-NA as a substrate). These findings suggest that depending on the esterase isoform, there may be different changes in the activity of this enzyme, and thus, either an increase (Bhatt et al., 2020) or decrease (present study) could be observed after exposing bees to pyrethroids.

Exposure of *O. bicornis* to Mospilan did not change EST activity. This is also consistent with the results of Delkash-Roudsari et al. (2022), who orally exposed *A. mellifera* to Confidor 200 SC (i.e., 0.16 μg/mL of imidacloprid as a.s.) for 2 h. Similarly, Zhu et al. (2020) demonstrated no changes in EST activity 2 days after exposure (via the single tower spray treatment) of *A. mellifera* to Advise 2 FL (i.e., 58.6 μg/mL of imidacloprid a.s.), although an earlier experiment by Zhu et al. (2017a) indicated that EST activity in *A. mellifera* increased by 50% following oral exposure for 14 days to Advise 2 FL (i.e., concentration of 0.92 μg/mL for imidacloprid as a.s.).

4.4. Effect of insecticides on ATP levels

We revealed significant effects of all three studied insecticides and several interactions between them, as well as between insecticides and sampling day, i.e., time, on ATP levels in *O. bicornis*. First, Dursban reduced the positive impact of Sherpa on ATP levels, and its impact strengthened with time after a single exposure. Dursban also reduced the positive effect of Mospilan on the ATP level. Furthermore, the presence of Dursban caused the disappearance of the antagonistic effect between Mospilan and Sherpa. While our findings concerning ATP are of significant interest, a direct comparison of our results in terms of the combined effects of insecticides on the ATP level in bees is impossible due to the lack of similar studies in the literature. In general, in organisms exposed to a stressor, low levels of ATP are expected, which can be associated with mitochondrial uncoupling (disruption of the proton gradient across the mitochondrial membrane), resulting in a slowdown of oxidative phosphorylation and ATP deficiency (Tiwari et al., 2002). However, we observed that ATP levels exhibited an increase with increasing concentration on the day after exposure to Sherpa, although this effect decreased both with time and with increasing Dursban concentration. Contradictory to our results, Bendahou et al. (1999) noticed that injecting honeybees with sublethal doses (0.4, 0.8, and 1 nmol/bee) of cypermethrin resulted in a decrease in ATPase activity within 3 h after exposure (bees were collected 0, 15, 30, 60, 120 and 180 min after injection). A decrease in ATP content was also observed in *Bombyx mori* larvae that were fed for one day with a sublethal dose of the pyrethroid λ-cyhalothrin at LC_{10} (0.21 μg/mL) applied to mulberry leaves (Ren et al., 2023). Additionally, Powner et al. (2016) demonstrated that ATP declined by 25% after exposing bumblebees (*Bombus terrestris audax*) to another neonicotinoid, imidacloprid (10 nM in 50% sucrose), for 10 days, following a recovery period of 22 days, whereas in our study, exposure to Mospilan (with acetamiprid a.s.), in the absence of Dursban and Sherpa, increased ATP levels.

Dursban and Mospilan exposure, in the absence of other insecticides, also raised ATP levels. Increased ATP levels were also observed in the

semen of *A. mellifera* drones after exposure to fipronil (0.001 μg/mL) for 20 days via contaminated syrup (Kairo et al., 2016). The authors pointed out that such an increase in ATP levels could be related not only to increased metabolism but also to decreased ATP consumption (Kairo et al., 2016). After exposure of *A. mellifera* bees to pollen contaminated with pesticide mixtures composed of a.s. of insecticides and fungicides at environmentally relevant concentrations (mixture 1: Cyrpodinil, Difenoconazole, Dodine, Fludioxonil, Tau fluvalinate; mixture 2: Chlorpyrifos ethyl, Cyrpodinil, Fludioxonil, Iprodione) decreased ATP levels were observed for bee abdomen, and at the same time, increased ATP levels were observed for bee thorax (Prado et al., 2020). Such differences in ATP levels between body parts could be attributed to compensatory changes, i.e., an increase in energy demand in one part of the body is compensated by a decrease in another (Prado et al., 2020). As suggested by Prado et al. (2020) and Schmitt et al. (2021), we also suppose that the increase in ATP levels observed in our study after exposure to Sherpa (without the presence of Dursban) or Mospilan (without the presence of Dursban), which were used at relatively low concentrations, was caused by the increased metabolic demand for detoxification processes upon exposure to the insecticide. Such an increase in ATP after exposure to various pesticides has been found for different invertebrates (Martelli et al., 2022; Schmitt et al., 2021). On the other hand, the presence of Dursban, which is the most toxic to *O. bicornis* among the studied insecticides (Mokkapati et al., 2021), ultimately impaired mitochondrial energy production. Thus, our findings suggest that the impact of insecticides on bee energy metabolism can vary depending on whether they are used alone or in combination, which may be influenced by their different modes of action.

5. Conclusion

This is the first study on non-*Apis* bees where the impact of three insecticide formulations at sublethal levels on bee biochemical processes was examined by assessing various biomarkers: three enzymatic (AChE, GST, EST) and one nonenzymatic related to energy metabolism (ATP). We showed that AChE and EST are suitable markers of exposure not only to organophosphates but also to pyrethroids, while GST seems to be a reliable marker for pyrethroids.

The most sensitive biomarker of exposure to the tested insecticides and their mixtures was ATP, which showed a complex pattern of response. Given that exposure to Sherpa and Dursban led to reduced AChE and EST activities, along with the increased ATP level in response to Sherpa exposure, a metabolic shift may have occurred: some metabolic pathways could become more active (ATP generation), while others might have been inhibited (decrease in enzyme activity) to maintain organism functioning despite the stress. The presence of Dursban in mixture impaired mitochondrial energy production, suggesting that the impact of insecticides on bee energy metabolism can vary depending on whether they are used alone or in combination, which may be influenced by their different modes of action. Dursban itself also caused the highest mortality of bees, even at concentrations much lower than recommended for field application. Thus, our results suggest that organophosphates should not be mixed with neonicotinoids and/or pyrethroids, as such combinations of insecticides negatively affect metabolism of solitary bees and increase their mortality.

In summary, this study provides insight into the effects of insecticide mixtures on sublethal endpoints in solitary bees, enhancing our understanding of the risks posed by pesticides to non-*Apis* bees.

Funding

This study was supported by the National Science Centre, Poland within SONATA 13 (2017/26/D/NZ8/00606) and by the statutory funds of the Institute of Nature Conservation, Polish Academy of Sciences. Access to research facilities was supported through the subsidy for scientific activity of Jagiellonian University (N18/DBS/000003).

CRediT authorship contribution statement

Anna Misiewicz: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zuzanna M. Filipiak:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Kamila Kadyrova:** Investigation. **Agnieszka J. Bednarska:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142233.

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SUPPLEMENTARY MATERIALS

Combined effects of three insecticides with different modes of action on biochemical responses of the solitary bee *Osmia bicornis*

Anna Misiewicz[1*], Zuzanna M. Filipiak[1], Kamila Kadyrova[2], Agnieszka J. Bednarska[1]

[1]Institute of Nature Conservation, Polish Academy of Sciences, A. Mickiewicza 33, 31-120 Kraków, Poland

[2]Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

[*]Corresponding author: email address: misiewicz@iop.krakow.pl

Table S1. Nominal concentrations of three Plant Protection Products Mospilan 20 SP, Sherpa 100 EC and Dursban 480 EC expressed as fraction of the recommended application concentration (×RAC) and concentrations of their active substances [mg/L], i.e., acetamiprid, cypermethrin and chlorpyrifos, respectively, used in full factorial experiment to study their effects on four biomarkers (acetylcholinesterase (AChE), glutathione S-transferase (GST), esterase (EST) activity [nM/min/mg protein] and ATP level [nM/mg body mass] in female *Osmia bicornis* bees after topical exposure. Number of bees analysed in different sampling days after initial exposure of bees is indicated.

Treatment	PPP			Active substance in PPP			Number of bees analysed												
	Mospilan 20SP	Sherpa 100EC	Dursban 480 EC	Acetamiprid	Cypermethrin	Chlorpyrifos	AChE				GST				EST				ATP		
							Sampling day														
		×RAC			mg/L		1	2	4	7	1	2	4	7	1	2	4	7	1	2	4
1*	0.0	0.0	0.0	0.0	0.0	0.0	5	5	5	5	5	3	5	5	5	4	5	5	5	5	5
2	0.5	0.0	0.0	40.0	0.0	0.0	5	5	4	5	5	5	5	5	5	5	5	5	5	5	5
3	1.0	0.0	0.0	80.0	0.0	0.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
4	0.0	0.0	0.2	0.0	0.0	192.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5	0.5	0.0	0.2	40.0	0.0	192.0	5	4	4	5	5	4	4	5	5	4	4	5	5	5	4
6	1.0	0.0	0.2	80.0	0.0	192.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
7	0.0	0.0	0.4	0.0	0.0	384.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
8	0.5	0.0	0.4	40.0	0.0	384.0	5	5	4	5	5	5	4	5	5	5	5	5	5	5	5
9	1.0	0.0	0.4	80.0	0.0	384.0	5	5	5	4	5	5	5	4	5	5	5	4	5	5	5
10	0.0	0.5	0.0	0.0	50.0	0.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
11	0.5	0.5	0.0	40.0	50.0	0.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
12	1.0	0.5	0.0	80.0	50.0	0.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
13	0.0	0.5	0.2	0.0	50.0	192.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
14	0.5	0.5	0.2	40.0	50.0	192.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
15	1.0	0.5	0.2	80.0	50.0	192.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
16	0.0	0.5	0.4	0.0	50.0	384.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
17	0.5	0.5	0.4	40.0	50.0	384.0	5	5	5	5	5	5	5	5	5	5	5	5	5	4	4
18	1.0	0.5	0.4	80.0	50.0	384.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
19	0.0	1.0	0.0	0.0	100.0	0.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
20	0.5	1.0	0.0	40.0	100.0	0.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
21	1.0	1.0	0.0	80.0	100.0	0.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
22	0.0	1.0	0.2	0.0	100.0	192.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
23	0.5	1.0	0.2	40.0	100.0	192.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
24	1.0	1.0	0.2	80.0	100.0	192.0	5	5	5	5	5	5	5	5	4	5	5	5	5	5	5
25	0.0	1.0	0.4	0.0	100.0	384.0	5	5	5	3	5	5	5	3	5	5	5	3	5	5	3
26	0.5	1.0	0.4	40.0	100.0	384.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
27	1.0	1.0	0.4	80.0	100.0	384.0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

*Control treatment with 0,01% Triton X-100

Table S2. Survival of female *Osmia bicornis* bees (together with total number of individuals used and sampled) exposed topically in two experiments run for the effect of Mospilan 20 SP, Sherpa 100 EC and Dursban 480 EC on enzyme (acetylcholinesterase, glutathione S-transferase, esterase) activities and ATP levels. Nominal concentrations of PPPs are expressed as fraction of the recommended application concentration (×RAC).

Treatment	Mospilan 20 SP ×RAC	Sherpa 100 EC ×RAC	Dursban 480 EC ×RAC	Number of individuals used	Number of individuals sampled*	Survival [%]
1**	0.0	0.0	0.0	50	50	100.0
2	0.5	0.0	0.0	50	50	100.0
3	1.0	0.0	0.0	50	48	96.0
4	0.0	0.0	0.2	50	47	94.0
5	0.5	0.0	0.2	50	40	80.0
6	1.0	0.0	0.2	50	49	98.0
7	0.0	0.0	0.4	68	41	60.3
8	0.5	0.0	0.4	65	38	60.6
9	1.0	0.0	0.4	61	34	57.2
10	0.0	0.5	0.0	50	49	98.0
11	0.5	0.5	0.0	50	47	94.0
12	1.0	0.5	0.0	51	46	90.4
13	0.0	0.5	0.2	50	45	90.0
14	0.5	0.5	0.2	52	44	84.9
15	1.0	0.5	0.2	50	46	92.0
16	0.0	0.5	0.4	52	35	68.0
17	0.5	0.5	0.4	50	36	72.0
18	1.0	0.5	0.4	51	40	79.3
19	0.0	1.0	0.0	52	46	89.0
20	0.5	1.0	0.0	50	47	94.0
21	1.0	1.0	0.0	50	47	94.0
22	0.0	1.0	0.2	50	38	76.0
23	0.5	1.0	0.2	50	46	92.0
24	1.0	1.0	0.2	51	45	88.5
25	0.0	1.0	0.4	55	31	57.1
26	0.5	1.0	0.4	55	39	71.0
27	1.0	1.0	0.4	47***	36	78.1

* Number of sampled bees is different than number of bees analysed in different sampling days (see Table S1), as not all bees that survived till sampling day were analysed for enzyme activity and ATP levels

** Control treatment with 0.01% Triton X-100

*** Bees that were unexpectedly lost were not included in the survival rate calculation

Table S3. Mean ± SD (standard deviation) values of acetylcholinesterase (AChE) activity [nM/min/mg protein] in female *Osmia bicornis* bees sampled at different days after topical exposure.

Treatment	PPP			Acetylcholinesterase activity [nM/min/mg protein]			
	Mospilan 20SP	Sherpa 100EC	Dursban 480 EC	Sampling Day			
		×RAC		1	2	4	7
1*	0	0	0	0.78±0.041	0.67±0.083	0.80±0.097	0.78±0.150
2	0.5	0	0	0.82±0.161	0.66±0.078	0.82±0.094	0.87±0.087
3	1	0	0	0.85±0.100	0.96±0.110	0.87±0.101	0.74±0.088
4	0	0	0.2	0.79±0.136	0.74±0.100	0.72±0.121	0.76±0.099
5	0.5	0	0.2	0.69±0.069	0.65±0.133	0.73±0.051	0.73±0.108
6	1	0	0.2	0.61±0.112	0.76±0.140	0.85±0.147	0.73±0.166
7	0	0	0.4	0.62±0.084	0.66±0.101	0.62±0.126	0.79±0.081
8	0.5	0	0.4	0.51±0.150	0.65±0.077	0.58±0.059	0.75±0.109
9	1	0	0.4	0.50±0.120	0.68±0.188	0.65±0.068	0.65±0.077
10	0	0.5	0	0.51±0.104	0.60±0.126	0.50±0.054	0.63±0.215
11	0.5	0.5	0	0.62±0.128	0.56±0.066	0.64±0.114	0.59±0.132
12	1	0.5	0	0.61±0.128	0.63±0.187	0.62±0.179	0.65±0.094
13	0	0.5	0.2	0.42±0.047	0.42±0.051	0.48±0.037	0.67±0.117
14	0.5	0.5	0.2	0.49±0.056	0.47±0.091	0.56/±0.205	0.68±0.124
15	1	0.5	0.2	0.54±0.094	0.47±0.051	0.53±0.124	0.55±0.070
16	0	0.5	0.4	0.50±0.097	0.44±0.078	0.53±0.108	0.50±0.128
17	0.5	0.5	0.4	0.61±0.150	0.62±0.133	0.57±0.076	0.54±0.193
18	1	0.5	0.4	0.63±0.122	0.69±0.112	0.54±0.045	0.61±0.127
19	0	1	0	0.79±0.111	0.67±0.084	0.80±0.133	0.63±0.238
20	0.5	1	0	0.87±0.179	0.63±0.113	0.69±0.102	0.80±0.265
21	1	1	0	0.73±0.064	0.74±0.139	0.58±0.094	0.71±0.176
22	0	1	0.2	0.68±0.118	0.66±0.163	0.67±0.079	0.57±0.178
23	0.5	1	0.2	0.51±0.097	0.56±0.098	0.55±0.083	0.69±0.126
24	1	1	0.2	0.57±0.109	0.62±0.037	0.58±0.043	0.55±0.129
25	0	1	0.4	0.56±0.106	0.57±0.059	0.66±0.157	0.69±0.241
26	0.5	1	0.4	0.56±0.108	0.60±0.093	0.54±0.096	0.53±0.041
27	1	1	0.4	0.45±0.123	0.58±0.101	0.56±0.094	0.49±0.096

*Control treatment with 0,01% Triton X-100

Table S4. Mean ± SD (standard deviation) values of glutathione S-transferase (GST) [nM/min/mg protein] in female *Osmia bicornis* bees sampled at different days after topical exposure.

Treatment	PPP			Glutathione S-transferase activity [nM/min/mg protein]			
	Mospilan 20SP	Sherpa 100EC	Dursban 480 EC	Sampling Day			
		×RAC		1	2	4	7
1*	0	0	0	420.6±91.51	447.8±57.15	354.2±71.03	340.1±62.09
2	0.5	0	0	446.8±191.87	430.7±109.35	422.6±68.62	375.3±78.40
3	1	0	0	445.8±39.07	428.7±88.54	369.3±87.19	397.5±53.60
4	0	0	0.2	424.7±129.00	401.5±96.09	386.4±73.03	385.9±79.39
5	0.5	0	0.2	337.1±35.04	398.7±99.39	358.5±96.38	403.5±123.68
6	1	0	0.2	465.9±93.36	504.2±131.99	376.4±74.92	383.4±144.01
7	0	0	0.4	410.6±137.91	404.9±155.59	448.8±114.31	377.4±57.37
8	0.5	0	0.4	477.0±175.18	451.8±109.51	476.7±95.59	426.7±103.63
9	1	0	0.4	408.6±138.22	385.4±119.42	378.9±76.88	381.1±95.06
10	0	0.5	0	468.9±112.13	486.0±47.55	396.9±67.44	463.9±83.77
11	0.5	0.5	0	436.7±119.46	536.4±81.02	456.9±67.82	589.7±67.26
12	1	0.5	0	463.9±63.18	463.9±83.09	423.6±58.40	570.6±71.74
13	0	0.5	0.2	440.8±70.49	548.4±102.99	520.3±131.33	428.7±61.97
14	0.5	0.5	0.2	455.8±119.73	437.7±152.86	419.6±81.41	462.9±127.09
15	1	0.5	0.2	396.5±68.93	430.7±127.22	437.7±27.79	539.4±152.71
16	0	0.5	0.4	485.0±77.18	593.7±130.33	534.3±92.60	465.9±124.91
17	0.5	0.5	0.4	457.9±116.81	528.3±83.97	482.0±66.12	527.3±64.37
18	1	0.5	0.4	479.0±67.45	410.6±124.15	416.6±159.48	532.3±91.50
19	0	1	0	640.0±113.03	533.3±74.54	453.8±100.40	473.0±96.39
20	0.5	1	0	386.4±20.89	570.6±97.34	492.1±98.49	459.9±68.31
21	1	1	0	511.2±128.65	407.5±61.11	463.9±103.93	358.2±174.13
22	0	1	0.2	457.9±125.94	382.4±115.01	440.8±116.63	444.8±94.91
23	0.5	1	0.2	578.1±163.56	510.2±105.94	474.0±89.04	514.2±150.67
24	1	1	0.2	679.2±75.64	502.1±101.22	414.6±75.27	416.6±196.66
25	0	1	0.4	485.0±225.45	408.6±132.52	532.3±69.84	434.4±187.20
26	0.5	1	0.4	457.9±83.59	475.0±76.69	525.3±132.10	568.6±150.86
27	1	1	0.4	533.3±102.19	503.1±169.40	558.5±157.55	524.3±93.95

*Control treatment with 0,01% Triton X-100

Table S5. Mean ± SD (standard deviation) values of esterase (EST) activity [nM/min/mg protein] in female *Osmia bicornis* bees sampled at different days after topical exposure.

Treatment	PPP			Esterase activity [nM/min/mg protein]			
	Mospilan 20SP	Sherpa 100EC	Dursban 480 EC	Sampling Day			
		×RAC		1	2	4	7
1*	0	0	0	1218.8±431.42	1214.6±420.36	1322.3±279.35	1078.1±332.25
2	0.5	0	0	1346.0±184.41	1353.8±299.24	1519.7±499.26	1575.8±560.99
3	1	0	0	1342.6±306.02	1289.3±360.12	1557.5±329.11	1279.3±256.25
4	0	0	0.2	1329.8±318.28	1413.3±386.33	1409.5±369.20	1447.6±187.02
5	0.5	0	0.2	1075.1±308.83	1758.6±236.99	1286.2±227.43	1398.2±391.29
6	1	0	0.2	966.7±182.82	1603.8±147.94	1264.6±223.21	1451.0±577.66
7	0	0	0.4	1169.4±208.99	1133.8±374.95	1050.1±252.60	1434.8±131.57
8	0.5	0	0.4	817.7±184.36	1259.1±239.93	869.4±227.87	1391.2±667.13
9	1	0	0.4	990.9±201.74	1282.8±300.98	1194.2±284.14	1326.4±577.43
10	0	0.5	0	892.4±269.54	1052.4±210.69	872.4±230.74	1070.0±514.92
11	0.5	0.5	0	866.6±227.78	812.6±213.33	955.9±383.54	942.8±246.53
12	1	0.5	0	697.1±450.38	959.1±261.99	1067.1±163.28	913.3±360.02
13	0	0.5	0.2	792.6±354.70	809.8±127.03	766.3±172.71	825.4±143.75
14	0.5	0.5	0.2	874.9±302.96	1031.6±248.19	849.0±636.68	1233.0±95.54
15	1	0.5	0.2	914.5±116.43	876.3±432.40	790.7±93.87	918.4±223.06
16	0	0.5	0.4	818.1±408.00	995.2±385.18	1035.6±307.73	856.7±132.86
17	0.5	0.5	0.4	954.3±255.92	1036.4±358.59	880.6±207.86	671.4±129.93
18	1	0.5	0.4	1232.4±181.43	1015.6±248.18	1038.4±124.88	1093.2±341.59
19	0	1	0	1031.6±447.80	962.0±113.81	937.5±271.77	940.0±213.28
20	0.5	1	0	1216.0±327.00	863.3±182.03	1092.0±262.19	1097.0±640.20
21	1	1	0	938.5±205.14	1071.9±344.16	981.7±277.74	984.9±497.29
22	0	1	0.2	1098.2±442.01	1033.2±96.96	897.2±331.16	900.3±216.64
23	0.5	1	0.2	811.0±225.00	877.9±223.13	874.6±157.46	1107.1±377.13
24	1	1	0.2	1145.6±424.21	1042.2±260.09	1023.7±314.37	909.3±195.43
25	0	1	0.4	906.1±123.18	939.7±329.15	1166.3±219.03	949.6±298.89
26	0.5	1	0.4	867.8±303.39	793.2±244.68	708.0±260.82	859.5±193.49
27	1	1	0.4	617.3±174.55	722.5±395.84	982.1±393.63	791.7±156.46

*Control treatment with 0,01% Triton X-100

Table S6. Mean ± SD (standard deviation) values of ATP level [nM/mg body mass] in female *Osmia bicornis* bees sampled at different days after topical exposure.

Treatment	PPP			ATP level [nM/mg body mass]		
	Mospilan 20SP	Sherpa 100EC	Dursban 480 EC	Sampling Day		
		×RAC		1	2	4
1*	0	0	0	61.6±22.85	133.3±26.73	111.3±35.20
2	0.5	0	0	139.4±67.53	153.6±40.34	218.9±91.54
3	1	0	0	129.7±72.62	164.0±53.43	159.0±62.42
4	0	0	0.2	171.6±91.16	181.2±48.53	148.9±66.57
5	0.5	0	0.2	137.4±67.09	176.6±5.88	215.9±71.01
6	1	0	0.2	146.9±60.69	154.0±50.30	155.4±73.06
7	0	0	0.4	123.5±65.98	211.6±70.81	165.8±33.77
8	0.5	0	0.4	141.0±78.97	139.9±55.25	161.3±74.16
9	1	0	0.4	145.1±73.79	163.8±68.78	181.7±182.64
10	0	0.5	0	263.5±75.33	182.2±43.40	152.5±51.09
11	0.5	0.5	0	158.5±98.63	188.1±65.67	140.7±26.03
12	1	0.5	0	179.1±101.65	193.6±49.00	150.1±38.02
13	0	0.5	0.2	225.2±59.21	220.0±50.88	137.9±46.61
14	0.5	0.5	0.2	334.9±75.52	133.1±20.64	161.2±21.52
15	1	0.5	0.2	163.8±98.23	156.9±41.71	171.6±32.43
16	0	0.5	0.4	156.6±73.04	174.7±35.51	152.3±12.31
17	0.5	0.5	0.4	194.8±66.85	166.4±40.38	161.1±37.91
18	1	0.5	0.4	127.9±50.55	255.3±118.18	175.4±34.07
19	0	1	0	201.8±29.66	402.0±129.67	141.1±27.16
20	0.5	1	0	197.3±51.26	354.2±210.69	191.9±46.63
21	1	1	0	155.5±49.62	330.5±125.78	131.7±39.86
22	0	1	0.2	136.7±40.49	183.8±42.33	178.3±38.34
23	0.5	1	0.2	154.9±60.51	272.7±103.32	174.7±45.01
24	1	1	0.2	155.5±35.68	306.7±157.86	183.3±44.59
25	0	1	0.4	138.4±42.16	170.0±23.59	136.3±11.50
26	0.5	1	0.4	136.7±22.26	299.3±171.66	161.4±36.72
27	1	1	0.4	135.3±23.69	264.7±86.62	195.0±65.40

*Control treatment with 0,01% Triton X-100

FIGURES

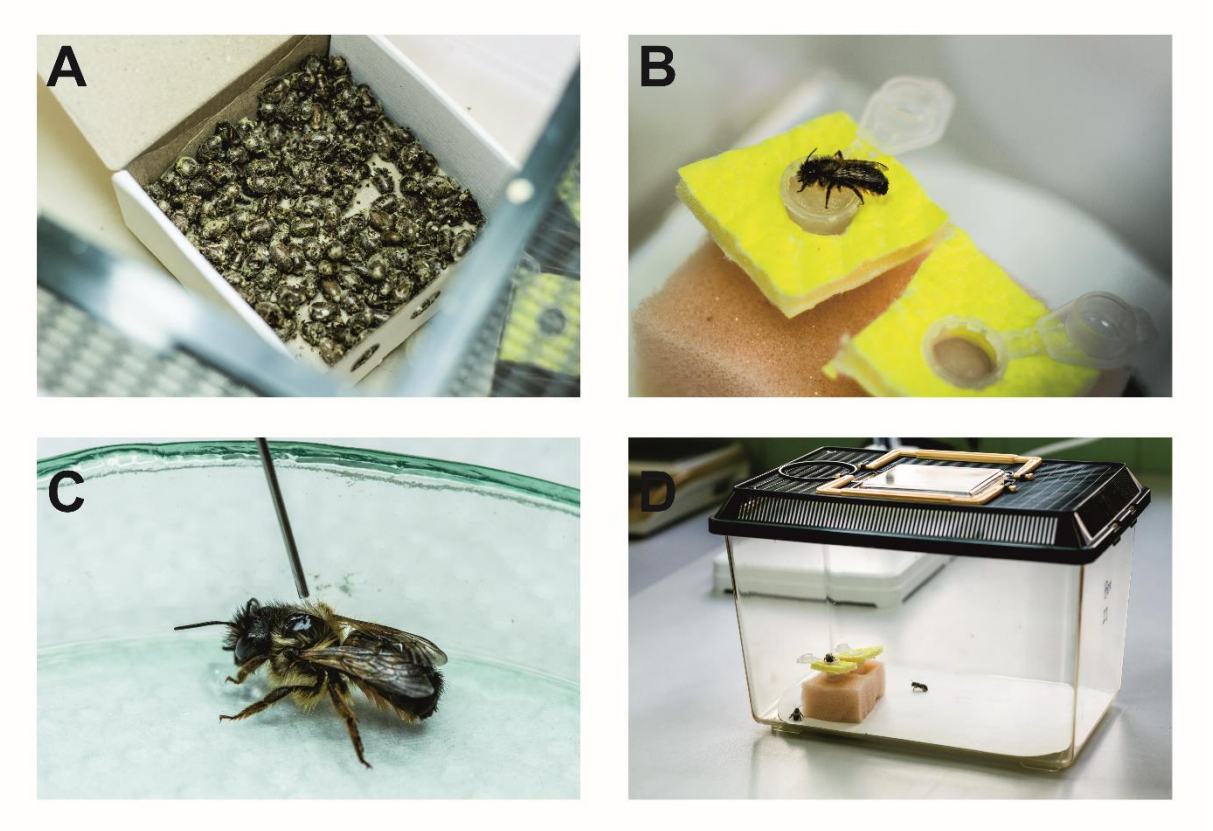

Figure S1. (A) Cocoons in cartoon box prepared for emergence; (B) Eppendorf tubes used to feed bees with 33% sucrose solution (w/w), with cotton wool inside to prevent bees from entering the tubes and a small piece of yellow sponge placed around the tube to attract bees to the food; (C) Topical application of the treatment solution to female bees on glass Petri dishes using a Hamilton micro-syringe with a repeating applicator; (D) Plastic box used for group housing of bees (min. 5 bees per treatment) after application of the treatment solution.

Kraków, 25/09/2024

mgr Anna Misiewicz
Instytut Ochrony Przyrody
Polskiej Akademii Nauk
al. Adama Mickiewicza 33
31-120 Kraków

OŚWIADCZENIE

Oświadczam, że w pracach:

Misiewicz, A., Mikołajczyk, Ł., Bednarska, A.J., 2022. Impact of oilseed rape coverage and other agricultural landscape characteristics on two generations of the red mason bee *Osmia bicornis*. *Agriculture, Ecosystems & Environment* 352: 108514. https://doi.org/10.1016/j.agee.2023.108514

mój udział wynosił 60% i polegał na opracowaniu metodologii badań, wykonaniu prac terenowych w drugim sezonie badań, wykonaniu pomiarów wszystkich analizowanych cech historii życiowej pszczół oraz eksperymentów laboratoryjnych, wykonaniu analiz statystycznych, interpretacji i wizualizacji wyników, przygotowaniu tekstu maszynopisu, poprawie maszynopisu zgodnie z uwagami recenzentów.

Misiewicz, A., Mikołajczyk, Ł., Bednarska, A.J., 2023. Floral resources, energetic value and pesticide residues in provisions collected by *Osmia bicornis* along a gradient of oilseed rape coverage. *Scientific Reports* 13, 13372. https://doi.org/10.1038/s41598-023-39950-5

mój udział wynosił 65% i polegał na opracowaniu metodologii badań, przeprowadzeniu prac terenowych oraz laboratoryjnych, wykonaniu analiz statystycznych, interpretacji i wizualizacji wyników, przygotowaniu tekstu maszynopisu, poprawie maszynopisu zgodnie z uwagami recenzentów.

Misiewicz, A., Zbrozek, M., Laskowski, R., Bednarska, A. J. Combined effects of insecticides on survival of the red mason bee *Osmia bicornis* - manuskrypt (w recenzji w Chemosphere)

mój udział wynosił 70% i polegał na pracach koncepcyjnych oraz opracowaniu metodologii badań, wykonaniu eksperymentów laboratoryjnych, wykonaniu analiz statystycznych, interpretacji i wizualizacji wyników, przygotowaniu tekstu maszynopisu.

Misiewicz, A. Filipiak, Z. M., Kadyrova, K., Bednarska, A. J., 2024. Combined effects of three insecticides with different modes of action on biochemical responses of the solitary bee *Osmia bicornis*. *Chemosphere* 359, 142233. https://doi.org/10.1016/j.chemosphere.2024.142233

mój udział wynosił 60% i polegał na udziale w pracach koncepcyjnych oraz opracowaniu metodologii badań, wykonaniu eksperymentów laboratoryjnych i pomiarów aktywności badanych enzymów, przeprowadzeniu analiz statystycznych, interpretacji i wizualizacji wyników, przygotowaniu tekstu maszynopisu oraz poprawie maszynopisu zgodnie z uwagami recenzentów.

Misiewicz Anna

Kraków, 26/09/2024

dr hab. Agnieszka Bednarska, prof. IOP PAN
Instytut Ochrony Przyrody
Polskiej Akademii Nauk
al. Adama Mickiewicza 33
31-120 Kraków

O Ś W I A D C Z E N I E

Oświadczam, że w pracach:

Misiewicz, A., Mikołajczyk, Ł., Bednarska, A. J., 2022. Impact of oilseed rape coverage and other agricultural landscape characteristics on two generations of the red mason bee *Osmia bicornis*. *Agriculture, Ecosystems & Environment* 352: 108514. https://doi.org/10.1016/j.agee.2023.108514
mój udział wynosił 25% i polegał na opracowaniu koncepcji badań oraz uczestniczeniu w opracowaniu metodologii badań, pomocy w wykonaniu analiz statystycznych oraz w przygotowaniu tekstu, udzielaniu krytycznych uwag i sugestii na wszystkich etapach powstawania pracy, oraz pozyskaniu funduszy na badania.

Misiewicz, A., Mikołajczyk, Ł., Bednarska, A. J., 2023. Floral resources, energetic value and pesticide residues in provisions collected by *Osmia bicornis* along a gradient of oilseed rape coverage. *Scientific Reports* 13, 13372. https://doi.org/10.1038/s41598-023-39950-5
mój udział wynosił 25% i polegał na uczestniczeniu w pracach koncepcyjnych, pomocy w wykonaniu analiz statystycznych, współudziale w przygotowaniu tekstu, udzielaniu krytycznych uwag i sugestii na wszystkich etapach powstawania pracy, oraz pozyskaniu funduszy na badania.

Misiewicz, A., Zbrozek, M., Laskowski, R., Bednarska, A. J. Combined effects of insecticides on survival of the red mason bee *Osmia bicornis* - manuskrypt (w recenzji w *Chemosphere*)

mój udział wynosił 20% i polegał na uczestniczeniu w pracach koncepcyjnych, pomocy w pracach laboratoryjnych i przy opracowaniu metodologii badań, pomocy przy wykonaniu analiz statystycznych, współudziale w przygotowaniu tekstu, udzielaniu krytycznych uwag i sugestii na wszystkich etapach powstawania pracy, oraz pozyskaniu funduszy na badania.

Misiewicz, A. Filipiak, Z. M., Kadyrova, K., Bednarska, A. J., 2024. Combined effects of three insecticides with different modes of action on biochemical responses of the solitary bee *Osmia bicornis*. *Chemosphere* 359, 142233. https://doi.org/10.1016/j.chemosphere.2024.142233

mój udział wynosił 15% i polegał na udziale w pracach koncepcyjnych, pomocy przy wykonaniu analiz statystycznych, współudziale w przygotowaniu tekstu, udzielaniu krytycznych uwag i sugestii na wszystkich etapach powstawania pracy, oraz pozyskaniu funduszy na badania.

Agnieszka Bednarska

Elektronicznie podpisany przez Agnieszka Bednarska
Data: 2024.09.26 09:35:07 +02'00'

Kraków, /06/2024

dr Łukasz Mikołajczyk
Instytut Ochrony Przyrody
Polskiej Akademii Nauk
al. Adama Mickiewicza 33
31-120 Kraków

O Ś W I A D C Z E N I E

Oświadczam, że w pracach:

Misiewicz, A., Mikołajczyk, Ł., Bednarska, A. J., 2022. Impact of oilseed rape coverage and other agricultural landscape characteristics on two generations of the red mason bee *Osmia bicornis*. *Agriculture, Ecosystems & Environment* 352, 108514. https://doi.org/10.1016/j.agee.2023.108514
mój udział wynosił 15% i polegał na uczestniczeniu w pracach koncepcyjnych dotyczących wyboru i analizy krajobrazu, przeprowadzaniu analiz charakterystyki krajobrazu i badanych stanowisk, wizualizacji danych dotyczących struktury krajobrazu oraz przygotowaniu tekstu w zakresie opisu krajobrazu.

Misiewicz, A., Mikołajczyk, Ł., Bednarska, A. J, 2023. Floral resources, energetic value and pesticide residues in provisions collected by *Osmia bicornis* along a gradient of oilseed rape coverage. *Scientific Reports* 13, 13372. https://doi.org/10.1038/s41598-023-39950-5
mój udział wynosił 10% i polegał na uczestniczeniu w opracowaniu metodologii badań, pomocy w wykonaniu pomiarów kolorymetrycznych pyłku oraz przygotowaniu wizualizacji danych dotyczących krajobrazu (mapa stanowisk).

Rochester, NY, USA, 25/09/2024

mgr Maryellen Zbrozek
Institute of Environmental Sciences
Jagiellonian University
ul. Gronostajowa 7
30-387 Kraków

CO-AUTORSHIP DECLARATION

I declare that in the manuscript:

Misiewicz, A., Zbrozek, M., Laskowski, R., Bednarska, A. J. Combined effects of insecticides on survival of the red mason bee *Osmia bicornis* - manuscript (under review in *Chemosphere*).

my contribution represents 5% and consisted of performing one laboratory experiment on the effects of the insecticides Karate Zeon 050 CS and Closer on solitary bees and reviewing the literature useful for discussing the results.

Maryellen Zbrozek

Kraków, 26/09/2024

prof. dr hab. Ryszard Laskowski
Instytut Nauk o Środowisku
Uniwersytet Jagielloński
ul. Gronostajowa 7
30-387 Kraków

O Ś W I A D C Z E N I E

Oświadczam, że w pracy:

Misiewicz, A., Zbrozek, M., Laskowski, R., Bednarska, A. J. Combined effects of insecticides on survival of the red mason bee *Osmia bicornis* – manuskrypt (w recenzji w *Chemosphere*)

mój udział wynosił 5% i polegał na uczestniczeniu w pracach koncepcyjnych, udzielaniu krytycznych uwag i sugestii na wszystkich etapach powstawania pracy oraz współudziale w przygotowywania tekstu.

(Prof. dr hab. Ryszard Laskowski)

Kraków, 18/06/2024

dr Zuzanna Filipiak
Instytut Nauk o Środowisku
Uniwersytet Jagielloński
Gronostajowa 7
30-387 Kraków

O Ś W I A D C Z E N I E

Oświadczam, że w pracy:

Misiewicz, A. Filipiak, Z. M., Kadyrova, K., Bednarska, A. J., 2024. Combined effects of three insecticides with different modes of action on biochemical responses of the solitary bee *Osmia bicornis*. *Chemosphere* 359, 142233. https://doi.org/10.1016/j.chemosphere.2024.142233

mój udział wynosił 20% i polegał na uczestnictwu w opracowaniu metodologii badań dotyczącej analizy esterazy, przeprowadzeniu pomiarów ATP, pomocy w wykonaniu analiz statystycznych, oraz współudziale w przygotowaniu tekstu.

Zuzanna Filipiak

Kraków, 19/06/2024

MSc Kamila Kadyrova
Institute of Environmental Sciences
Jagiellonian University
ul. Gronostajowa 7
30-387 Kraków

CO-AUTORSHIP DECLARATION

I declare that in the manuscript:

Misiewicz, A. Filipiak, Z. M., Kadyrova, K., Bednarska, A. J., 2024. Combined effects of three insecticides with different modes of action on biochemical responses of the solitary bee Osmia bicornis. ***Chemosphere*** **359, 142233.** https://doi.org/10.1016/j.chemosphere.2024.142233

my contribution was 5% and consisted of participation in performing measurements of AChE activity in 9 of the 26 treatments and reviewing the literature useful for discussing the results.

Kamila Kadyrova