Pesticide contamination in nestlings of Montagu's harrier, *Circus pygargus*: patterns, factors of influence and sublethal effects

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“The solution to pollution is not dilution”

Barry Commoner, *The Closing Circle* (1971)

Acknowledgements – Remerciements

Voilà un moment que tous les doctorant(e)s attendent avec impatience: le temps d'écrire les remerciements. Dans mon cas, il s'agit des dernières lignes que j'ajoute à ce manuscrit. J'ai attendu d'avoir été au bout de ce travail pour le boucler avec émotion.

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Maintenant, je vais passer quelques lignes à remercier les personnes qui ont fait de mes trois années de thèse au CEBC des années très spéciales. Merci aux services communs d'analyses biologiques, de l'administration, de l'informatique, de l'entretien, de l'atelier et du restaurant pour leur travail au quotidien. Vous avez une place centrale au CEBC alors merci pour l'aide que vous avez pu m'apporter directement ou indirectement. Merci notamment à Delphine et Cécile pour l'aide que vous m'avez apporté pour mes demandes de mission ou pour préparer mes aliquots de PBS, mais aussi pour avoir organisé avec les lutins du CAES tant d'événements au labo ! Un remerciement spécial aussi à Arnaud et Morgane, nos deux chefs, sans qui je n'aurais certainement pas mangé grand-chose à part des pâtes… Votre cuisine est un vrai délice et je vous remercie d'intégrer de plus en plus de plats pour les herbivores comme moi ! Mes papilles vont vous regretter. Enfin, merci à la direction du CEBC, en particulier Charly, dont la porte a toujours été ouverte pour régler des galères de dernière minute et dont la bienveillance m'a toujours fait chaud au cœur.

Merci à l'équipe Résilience au sein de laquelle j'ai pu évoluer au cours de ces trois années. Le grand turnover m'a permis de côtoyer de nombreuses personnes dont je ne saurais citer tous les noms mais qui ont toutes ajoutées une pierre à l'édifice des dissidents. Au sein de cette équipe, plusieurs ornithos se sont succédés et ont tous contribué à la réussite de cette thèse car ce sont eux, les bras de l'ombre, qui ont œuvré pour le suivi des bubus depuis de nombreuses années. Malheureusement, je ne connais pas tous leurs noms mais je peux tout de même remercier ceux que j'ai côtoyés pendant mes 3 saisons de terrain : Samuel, Guillaume (alias Guigui), Bastien, Clotilde, Gildas et Dorian (+Etienne pour les captures d'adultes). Merci de m'avoir formée puis appuyée sur le terrain et de m'avoir fait partager votre passion dévorante pour les oiseaux. Merci à JLG également d'avoir permis la découverte de dizaines de nids grâce à son super drône ! Merci également à Juliette Rabdeau, ma prédécesseure que je n'ai malheureusement pas connu mais qui a permis, sans même le savoir à l'époque, d'avoir des données précieuses pour cette thèse. Je remercie également les stagiaires que j'ai pu encadrer et qui m'ont grandement aidé sur le terrain et au labo. Moran, Camille, Noah, Olivia et Jonathan, j'espère que vous garderez de bons souvenirs de votre passage au CEBC. Un grand merci à Sorenza aussi pour m'avoir immortalisé à plusieurs reprises les moments vécus sur le terrain. C'est grâce à toi qu'il y a de si beaux clichés dans cette thèse et que mon film a pu être un succès ! Merci aussi d'être toujours de bonne compagnie, dans les bons moments comme dans les galères. Je suis heureuse de te compter parmi mes amies.

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Je ne pourrais continuer ces remerciements sans parler de la personne qui m'a accueillie et intégrée au sein de l'équipe et du labo : Agathe. Merci d'avoir été là pour m'accompagner dans mes premiers pas de doctorante, pour m'avoir tout de suite montré ce qu'était la grande famille du CEBC et pour avoir partagé des moments importants dans ma vie. Je te serais toujours reconnaissante car cette aventure n'aurait pas été la même sans toi. Tu as su me guider dans les méandres des commandes de matériel et dans la rigueur (voire maniaquerie) des manips, mais aussi dans l'art de vivre à la campagne (le comble pour une parisienne !). Lorsque j'ai adopté Salto tu as craqué et adopté Séthi, et ces deux là sont devenus les meilleurs amis du monde (même si peuchère Séthi il subit beaucoup Salto). Nous avons partagé beaucoup de bons moments avec ces deux là et je te remercie pour avoir pris soin de mon « fils » à de multiples reprises. Les séjours chez tata lui manquent mais il sait qu'il vous reverra bien vite au bord d'une plage normande !

En parlant d'une super tata, je ne peux oublier Pauline ! Je te remercie d'avoir pris soin de ma petite tribu, en particulier pendant mes nombreuses absences de cette dernière année… Tu as été là pour eux mais aussi pour moi. Je te remercie pour nos discussions autour d'un bon coteaux-du-Layon ou simplement autour d'un thé. Ma chère Pauline, tu as tellement égayé les journées au labo avec ton sourire et ton rire si caractéristiques que tu as laissé un grand vide en partant de l'autre côté de l'Atlantique. Tu me manques mais je sais que nous nous reverrons. En attendant je vous envoie des bisous à Pépinot et toi. Et comment parler de Pauline sans parler d'Antonin ? Merci Anto d'être une oreille attentive et d'apporter toujours des conseils avisés et bienveillants. Vous êtes tous les deux des personnes merveilleuses que je garde précieusement dans mon cœur.

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Enfin, je terminerais mon tour des remerciements par ma famille. Parce que pour moi les animaux sont une partie intégrante de la famille, je remercie Prune, Nesquik, Léva et Salto, pour le soutien émotionnel au quotidien. Ils m'ont aussi amené de grosses galères mais la vie ne serait pas la même sans eux. Pour les autres membres de ma famille, je sais qu'ils ne liront pas non plus ce manuscrit entièrement et qu'ils ont sûrement encore du mal a décripter ce que je fais sur les oiseaux (comme pour toutes les personnes qui font de la recherche non ?) mais je tiens à les remercier. Bien que j'ai vécu loin d'eux depuis de nombreuses années, ils ont toujours été mon phare au milieu des tempêtes. Merci à ma cousine Stéf pour son soutien infaillible et à sa maman Silvia qui est ma deuxième maman finalement. Merci à tou(te)s mes cousin(e)s, mes tantes et oncles par alliance, et à JC pour les nombreuses fêtes partagées et leur soutien tout au long de ces trois années. Maman, je te remercie de m'avoir toujours soutenue dans ma vie, et malgré la particularité de notre situation je sais que je peux compter sur toi, peu importe le nombre de kilomètres qui nous séparent. Si tu savais combien je t'aime et comme je t'admire. Merci à mes frères aussi, car c'est vous qui me donnez la force d'avancer dans les moments difficiles alors merci. Mes petits frères, je vous aime.

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PREFACE

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The present manuscript is the result of my research work, which started on October 4th, 2021, and ended on October 22nd, 2024, the day of submission. This work was realised as part of the PestiStress research program, which K. Monceau, my thesis director, handled. My doctoral thesis was therefore partly funded by the research national agency (ANR) JCJC PestiStress (grant #19-CE34-0003-01) and by the region Nouvelle-Aquitaine (grant BioBird). During this three-year work, I was part of the Résilience team from the Centre d'Études Biologiques de Chizé (UMR7372 CNRS & La Rochelle Université, USC1339 INRAE, CNRS & La Rochelle Université) and of the doctoral school Euclide from La Rochelle Université.

This work is based on data collected from a long-term monitoring program of the Montagu's harrier *Circus pygargus* population of the Zone Atelier Plaine & Val de Sèvre (ZAPVS). Therefore, I benefited from the data collected between 2016 and 2021, before the start of my doctoral work. Nonetheless, I completed the dataset through the examination under the microscope of the blood smears collected in 2019, 2020 and 2021. I used the available data to conceive the studies presented in the manuscript. Besides, I participated in data collection during fieldwork seasons (generally lasting from May to August) in 2022, 2023 and 2024. During these periods, I searched nests in the 450-km^2 study area, along with ornithologists of the Résilience team and volunteers, and monitored nests during both the laying period and rearing period, along with at least one ornithologist in the team. I handled nestlings to perform data and sample collection during the last nest visit of the three fieldwork seasons, thanks to the certification to use wild fauna to conceive and realise experimentations for scientific purposes that I obtained in 2022 (provided by the Museum National d'Histoire Naturelle (MNHN)). After fieldwork, I managed sample processing (centrifugation of blood samples, fixation and staining of blood smears) and storage before laboratory analyses. I also assisted M. Teixeira at the Biogéosciences laboratory in 2024 for the laboratory analyses of the samples collected in 2023 (titration of carotenoids and acetylcholinesterase). However, I did not participate in the pesticide titration, entrusted to M. Millet at the ICPEES laboratory. All other activities that I realised during my three-year work (teaching, supervising, communications, etc.), are listed in this "Preface" section.

The core of this manuscript is composed of a general introduction, a section on general methods, and three chapters that include five articles that I wrote as the first author (four published and one in preparation). Three other articles are mentioned in the manuscript and presented in the appendices, one published that I wrote as a first author (along with its corrigendum) and two under review that I co-authored. These articles are listed hereafter. A general discussion of my research work is provided and ends with a general conclusion. The references used to write the articles and appendices are directly included after the corresponding section while all references mentioned in the general introduction, methods, and discussion are listed after the general discussion.

I wish you a pleasant reading!

I. Publications

This manuscript is based on the following publications, which are included as a chapter's part or as appendices:

- **Fuentes, E.**, Gaffard, A., Rodrigues, A., Millet, M., Bretagnolle, V., Moreau, J., Monceau, K., 2023. Neonicotinoids: Still present in farmland birds despite their ban. *Chemosphere*, 321, 138091. https://doi.org/10.1016/j.chemosphere.2023.138091
- **Fuentes, E.**, Gaffard, A., Rodrigues, A., Millet, M., Bretagnolle, V., Moreau, J., Monceau, K., 2023. Corrigendum to" Neonicotinoids: Still present in farmland birds despite their ban, Chemosphere, 321, April 2023, 138091". *Chemosphere*, 325, 138344. https://doi.org/10.1016/j.chemosphere.2023.138344
- **Fuentes, E**., Moreau, J., Teixeira, M., Bretagnolle, V., Monceau, K., 2023. Effects of conventional vs. organic farming practices on raptor nestling health: neither black nor white. *Agriculture, Ecosystems & Environment*, 358, 108719. https://doi.org/10.1016/j.agee.2023.108719
- **Fuentes, E**., Moreau, J., Rodrigues, A., Millet, M., Bretagnolle, V., Monceau, K., 2024. Pesticide contamination patterns in Montagu's harrier (*Circus pygargus*) chicks. *Environmental Science and Pollution Research*. https://doi.org/10.1007/s11356-024-34937-9
- **Fuentes, E**., Moreau, J., Millet, M., Bretagnolle, V., Monceau, K., 2024. Organic farming reduces pesticide load in a bird of prey. *Science of the Total Environment*, 930, 172778. https://doi.org/10.1016/j.scitotenv.2024.172778
- **Fuentes, E.**, Moreau, J., Millet, M., Bretagnolle, V., Monceau, K., 2024. First evidence of age-dependent decreases in non-persistent pesticide mixtures in nestlings of a farmland raptor. *Environmental Research*, 263, 120179. https://doi.org/10.1016/j.envres.2024.120179
- **Fuentes, E.**, Moreau, J., Millet, M., Bretagnolle, V., Monceau, K. Pesticide mixture effects on physiological stress and morphology of growing wild nestlings. In preparation for *Journal of Hazardous Materials*.
- Bariod, L., **Fuentes, E**., Millet, M., White, J., Jacquiod, S., Moreau, J., Monceau, K. First field evidence of pesticide-based intestinal microbiota differences. Under review in *Environment International*.
- Bariod, L., **Fuentes, E**., Millet, M., Jacquiod, S., White, J., Moreau, J., Monceau, K. Direct and indirect effects of pesticide exposure on farmland raptor gut microbiota. Under review in *Journal of Hazardous Materials*.

II. Publications from side projects

The publications listed hereafter are not included in the manuscript as these were unrelated to the research work of my thesis.

- **Fuentes, E.**, Lecardonnel, M., Triplet, P., 2022. Methods of prioritizing waterbird species to improve the management of a protected area. *Alauda*, 90, 113-126.
- Masse, E., Drossart, P., **Fuentes, E.**, Sueur, F., Triplet, P., 2023. Use of space and time by the Eurasian Curlew *Numenius arquata* during the winter period. *Alauda*, 91, 17-26.
- Triplet, P., Cazanas, J., Bernard, C., **Fuentes, E.**, 2024. La prédation sur les oiseaux d'eau nicheurs, constats et réflexions sur la gestion. *Plume de naturalistes*, 8, 169-226.

III. Participations to scientific congress

Oral communications

"Conventional vs. organic farming effects on raptor nestling health: neither black nor white", November 2022. International Conference on Ecological Sciences SFE²-GfÖ-EEF, Metz, France.

"Effects of pesticides on Montagu's harrier, *Circus pygargus*, chicks", September 2023. Colloque des doctorants de La Rochelle Université, La Rochelle, France.

"Patrons de contamination aux pesticides des poussins de Busard cendré (*Circus pygargus*)", May 2023. 51ème congrès du Groupe Français de recherches sur les Pesticides (GFP), Paris, France.

"Patrons de contamination aux pesticides des poussins de Busard cendré (*Circus pygargus*)", July 2023. Congrès Petit Pois Déridé, Poitiers, France.

"Pesticide contamination of a bird of prey: variability and organic farming influence", November 2023. 6ème Colloque d'Écophysiologie Animale (CEPA), Tours, France.

"Pesticide contamination of a bird of prey: variability and organic farming influence", November 2023. 8ème Colloque du Réseau Ecotox, La Rochelle, France.

Poster presentations

"Neonicotinoid contamination in Montagu's harrier (*Circus pygargus*) chicks", September 2023. Colloque des doctorants de La Rochelle Université, La Rochelle, France.

"Changes in pesticide contamination with age in chicks of a bird of prey", May 2024. Europe 34th Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC), Seville, Spain.

IV. Vulgarization and other scientific involvement

1. Oral communications

"L'agriculture biologique comme levier de conservation pour le Busard cendré ?", October 2022. Festival International du Film Ornithologique (FIFO) de Ménigoute, Ménigoute, France.

"L'agriculture biologique comme levier de conservation pour le Busard cendré ?", March 2023. 23ème rencontres Réseau Busards, Melle, France.

2. Educational activities

Intervention for students of 3ème during their observation internship at the CEBC laboratory. February 2022 and January 2023, Villiers-en-bois, France.

Animation for scholar groups and public for the Fête de la Science: "Les rapaces du milieu agricole : le Busard cendré et ses menaces", October 2022, Villiers-en-bois, France.

Intervention Science en Mouvement d'Elle (SEME) from the Espace Mendès France for students from Collège François Albert: "À la découverte du monde de l'entreprise et de la recherche", March 2023, Celles-sur-Belle, France.

Animation for scholar groups for the Fête de la Science: "Jeu du Busard cendré", October 2023, Villiers-en-bois, France.

Scientific interviews at the University library "Intensification des pratiques agricoles: comment préserver les rapaces sauvages ?", March 2024, La Rochelle, France.

Animation for public for the Journée de la Conservation: "La Conservation du Busard dans les plaines agricoles", May 2024, Villiers-en-bois, France.

3. Media outreach

Interview from IFFCAM (Institut Francophone de Cinéma Animalier de Ménigoute) students, April 2022. https://www.youtube.com/watch?v=t3-pV9T5Uzg

Movie « Et si demain… » for the Festival du Film [Pas Trop] Scientifique from the ADocs of La Rochelle Université – Price of the best scientific vulgarization, November 2023. https://www.youtube.com/watch?v=SZK8IV2n1mE

Podcast « Il Etait une Thèse » from the ADocs and CMI Alumni Association of La Rochelle Université, January 2024. https://podcast.ausha.co/il-etait-une-these/

Contest « Ma Thèse en 180 secondes » from La Rochelle Université, March 2024. https://www.youtube.com/watch?v=gfO5q-xg6L0&t=3272s

4. Involvement in laboratory life

I co-supervised the planning, organization and communication of the laboratory's scientific seminars from December 2021 until December 2022.

I was member of the Equality, Diversity, Inclusion and Well-being committee since February 2022.

I was part of the laboratory council as representative of the doctoral and post-doctoral group since June 2023.

5. Reviewing activity

I reviewed a manuscript in July 2024 for the journal *Environmental Research*.

V. Teaching and supervising

During my thesis, I combined my research activity with teaching at La Rochelle Université, which represents a total of 112.5 hours of teaching, allocated as detailed below.

In 2022:

- Practical research internship in Chizé, Master 1 GEEL - 8h Supervised group project.
- Plant physiology, BSc L2 SV - 20h Practical work (TPs).

In 2023:

- Practical research internship in Chizé, Master 1 GEEL - 11h Supervised group project.
- Ecology, BSc L2 SV - 6h Supervised work (TDs).
- Genetics, BSc L1 SV - 3h Tutored work (TEA) and 15h TDs.
- Animal Biology, BSc L2 SV - 12h TPs.
- Plant Biology, BSc L1 SV – 13.5h TDs.

In 2024:

- Ecology, BSc L2 SV - 6h TDs.
- Genetics, BSc L1 SV - 3h TEA and 15h TDs.

I also supervised five interns during their Bachelor and Master Degree internships:

- Camille Arderius, BSc L2 SV of La Rochelle Université, during 4 weeks in June-July 2023.
- Noah Bonneau, BSc L2 SV of La Rochelle Université, during 4 weeks in June-July 2023.
- Moran Tomozyk, Master 1 GEEL of La Rochelle Université, during 8 weeks in April-May-June 2024.
- Jonathan Comby, BSc L2 SV of La Rochelle Université, during 4 weeks in June-July 2024.
- Olivia Bosson, BSc L2 SV of La Rochelle Université, during 4 weeks in June-July 2024.

VI. Courses

I attended the following courses during my thesis:

- Application of Wildlife Toxicology Studies to Support of Environmental Risk Assessment of Pesticides. Provided by Manuel Ortiz-Santaliestra, Nico van den Brink, Johan Axelman, Ana Lopez-Antia, Ralf Dittrich, John E. Elliott and Marta Villar. Theoretical course at the SETAC Europe 34th Annual Meeting.
- Wildlife experimentation: Training for the use of unhoused wildlife for scientific purposes. Provided by the Museum National d'Histoire Naturelle. Synchronous distance theoretical courses and practical training at the Centre d'Études Biologiques de Chizé.

- Applied statistics with R: Statistical modelling. Provided by David Pinaud. Theoretical courses and practical training at the Centre d'Études Biologiques de Chizé.
- Getting started with QGIS software. Provided by David Pinaud. Theoretical courses and practical training at the Centre d'Études Biologiques de Chizé.
- Introduction to scientific integrity. Provided by the URFIST network. Synchronous distance theoretical course.

GENERAL INTRODUCTION

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Most of the scientific community now acknowledges that the 6th biodiversity crisis is driving many species to extinction (Barnosky et al., 2011; Cowie et al., 2022). This ongoing 6th mass extinction is characterised by rapid environmental changes induced by human activities. Among the most common "human-made" drivers of species' decline are direct exploitation of species, land use change, climate change, invasive alien species and pollution, sometimes interacting in synergy, increasing the species' extinction risk (Sánchez-Bayo & Wyckhuys, 2019; Brook et al., 2008). Modern agriculture is responsible for worldwide land-use changes and pollution, notably with greenhouse gases contributing to climate change (Tubiello et al., 2015) and synthetic inputs contaminating ecosystems (Green et al., 2005). Consequently, agriculture has been identified as (one of) the leading driver(s) of biodiversity loss worldwide (Maxwell et al., 2016; Kehoe et al., 2017). Nonetheless, agriculture is still expected to intensify and expand rapidly as the global crop demand is estimated to double by 2050 (Tilman et al., 2011). Its consequences on biodiversity are thus projected to also increase rapidly (Kehoe et al., 2017).

I. Agriculture and biodiversity

1. History of agriculture

Since its appearance about 10,000 years ago in the Fertile Crescent of Mesopotamia, agriculture has induced land-use changes to convert natural habitats into exploitable fields for crop production (Tudi et al., 2021). For millenniums, agriculture has been hard work for low food supplies, and although farming practices never stopped evolving to be labour-saving, it is only in the last century that farming practices have undergone a rapid development that revolutionised agriculture (Zimdahl, 2015). As the Industrial Revolution proceeded, mechanised agriculture became more efficient, and the use of fertilisers boosted production (Zimdahl, 2015). In the aftermath of the world wars, because European nations were struggling to pay for imports of food and other necessary commodities, the USA launched a program of economic assistance for European recovery in 1947. This so-called Marshall Plan committed the Europeans to plan their own economic recovery, notably by adopting policies that favoured trade and increased production (Tarnoff, 2018). The objective of agriculture production for human consumption was to exceed pre-war levels by 15% by 1951, however, this was only achieved by 11%. Meanwhile, the rapid increase in human population has led to Europe's inability to feed itself (Tarnoff, 2018). In 1957, some governments (Germany, France, Italy and the Benelux countries) bound to a Common Agricultural Policy (CAP) to meet food requirements and reduce poverty among food producers (Donald et al., 2002). In the USA, similarly, farmers were pushed to "get big or get out" to feed the increasing population and to increase yields (Zimdahl, 2015). This led agriculture to undergo an intensification notably characterised by larger agricultural machines and the use of chemicals to increase productivity (Primdahl, 1990). This caused farmers to remove hedges and other natural elements to increase field size and allow the use of larger machines, which resulted in landscape homogenisation (Primdahl, 1990; Jongman, 2002; Vitikainen, 2004; Brown & Shulte, 2011; **Figure 1**). Overall, this led to the disappearance of small family farms in favour of large intensive farms, doubling their average size in 50 years (216 acres in 1950 to 434 acres in 2000 in the USA, Zimdahl, 2015). The Green Revolution, started in the 1960s also promoted the intensification of agricultural production in developing countries, notably through the share of US agriculture technology such as hybrid seeds also called modern high-yielding crop varieties (Evenson & Gollin, 2003; Patel, 2013). This accounted for almost 50% of yield growth and 40% of production growth for all developing countries between 1981 and 2000 (Evenson & Gollin, 2003).

Figure 1. Landscape homogenisation between 1950 (left) and 2024 (right) of an agricultural landscape located in Deux-Sèvres department, France. These aerial photographs have been taken at the same location and altitude above the villages of Juscorps (bottom) and Fors (top-left). The urban expansion, disappearance of hedgerows, reduction in the number of fields and an increase in their surface area can be seen. Source: IGN, 2024 (available at: https://remonterletemps.ign.fr/ accessed on September 2024).

2. Consequences and flaws of conventional agriculture

An intensive conventional agriculture is therefore characterised by homogeneous landscapes of large monocultures cultivated with a heavy mechanisation, and massive inputs of fertilizers and pesticides. Within the few decades of agricultural intensification, the drastic changes that occurred in farmlands led to rapid effects on biodiversity. A collapse in insects and farmland birds' populations was observed over the same period agriculture intensified, leading scientists to conclude a causal link (Krebs et al., 1999; Donald et al., 2001; Hallmann et al., 2017).

The conversion of land-use directly results in habitat loss for wild species, reducing local terrestrial biodiversity (Newbold et al., 2016). Intensive land-uses with a simplification and homogenization of landscapes further reduce the habitats and food resources available for a large number of species (Benton et al., 2003; Stanton et al., 2018). For instance, when hedgerows are scarce or smaller, lower diversity of herbivorous insects, lower breeding densities of birds, and lower abundances of small mammals are observed (de Bruijn, 1994; Holland & Fahrig, 2000; Gelling et al., 2007). Many bees, moths or beetles have lost floral resources, host plants, nesting and hibernation sites, which has been a determining factor in their decline (Sánchez-Bayo & Wyckhuys, 2019). In turn, this affects insectivorous birds as fewer prey biomass reduces their reproductive success (Jiguet, 2002).

A direct consequence of the mechanization of agricultural practices on species is the destruction of ground-nesting birds and their nests (Stanton et al., 2018). In species such as the Montagu's harrier *Circus pygargus* and the little bustard *Tetrax tetrax*, up to 55% of the clutch failures and ~50% of the loss in nest productivity are due to harvesting alone (Santangeli et al., 2015; Bretagnolle et al., 2018a). Mowing or harvesting might also have subsequent indirect effects as nests become more exposed to predators, leading to higher nest failures by predation (Giuliano & Daves, 2002; Santangeli et al., 2015). Nests might also be abandoned due to the disturbances linked to farmers' work (Assandri et al., 2017).

The intensive use of synthetic inputs may have both indirect and direct impacts on species and, thus, on biodiversity in agroecosystems (Sánchez-Bayo & Wyckhuys, 2019; Mitra et al., 2021; Wagner et al., 2021). For instance, fertilisers might induce the eutrophication of watercourses and lakes that change communities and might affect the whole ecosystem through cascade effects (Jenderedjian et al., 2007; Clark et al., 2017). Fertilisers increase net primary production and increase the nutrient content of plants but also decrease the plant species diversity by enhancing the growth of competitive species at the expense of slower-growing ones, which in turn affects grassland invertebrates (Vickery et al., 2001). Synthetic fertilisers have been recognised as a key driver of pollinator losses in Europe as they led to higher productivity of grass and crops and a lack of crop rotation, both at the expense of wildflowers (Ollerton et al., 2014). Earthworms and other invertebrates are directly vulnerable to fertiliser applications as some substances might be toxic and thus reduce their number in soils (Vickery et al., 2001). Similarly, synthetic pesticides might have direct effects (due to their toxicity) and indirect effects on non-target organisms (see details in the section "*II.4. Effects on non-target organisms*").

Alternative production systems arose to counteract the adverse effects of conventional farming on agroecosystem health. Organic farming, agroforestry, integrated farming, conservation agriculture, mixed crops and livestock, and innovative production systems are all alternatives to feed the planet safely while preserving ecosystems (Reganold & Wachter, 2016).

3. Organic vs. conventional farming

Organic farming is a production system that does not use synthetic pesticides or fertilisers to grow crops (Lockeretz, 2007; EPA, 2024a). Often considered inefficient and speculated to rely on larger lands to produce the same amount of food, organic farms and lands have steadily increased since the late 1970s (Lockeretz, 2007; Reganold & Wachter, 2016). Organic farms are highly efficient and economically competitive while promoting social well-being and being more environmentally friendly than neighbouring conventional farms (**Figure 2**; Lockeretz et al., 1981; Reganold & Wachter, 2016). Organic systems notably use less energy per hectare than conventional counterparts use and have higher soil organic matter, which makes them good candidates to limit fossil fuel emissions and store carbon in soils, both being important to counteract climate change (Reganold & Wachter, 2016). Organic farming systems encompass a large variety of practices that go beyond organic certification guidelines (which may vary slightly between countries), resulting in higher natural and semi-natural areas and soil quality under organic farming systems than under conventional systems (Gibson et al., 2007; Norton et al., 2009; Reganold & Wachter, 2016). This increases shelters and food resources available for insects and small mammal species, which, in turn, positively affect predators feeding on them (Hole et al., 2005; Tuck et al., 2014).

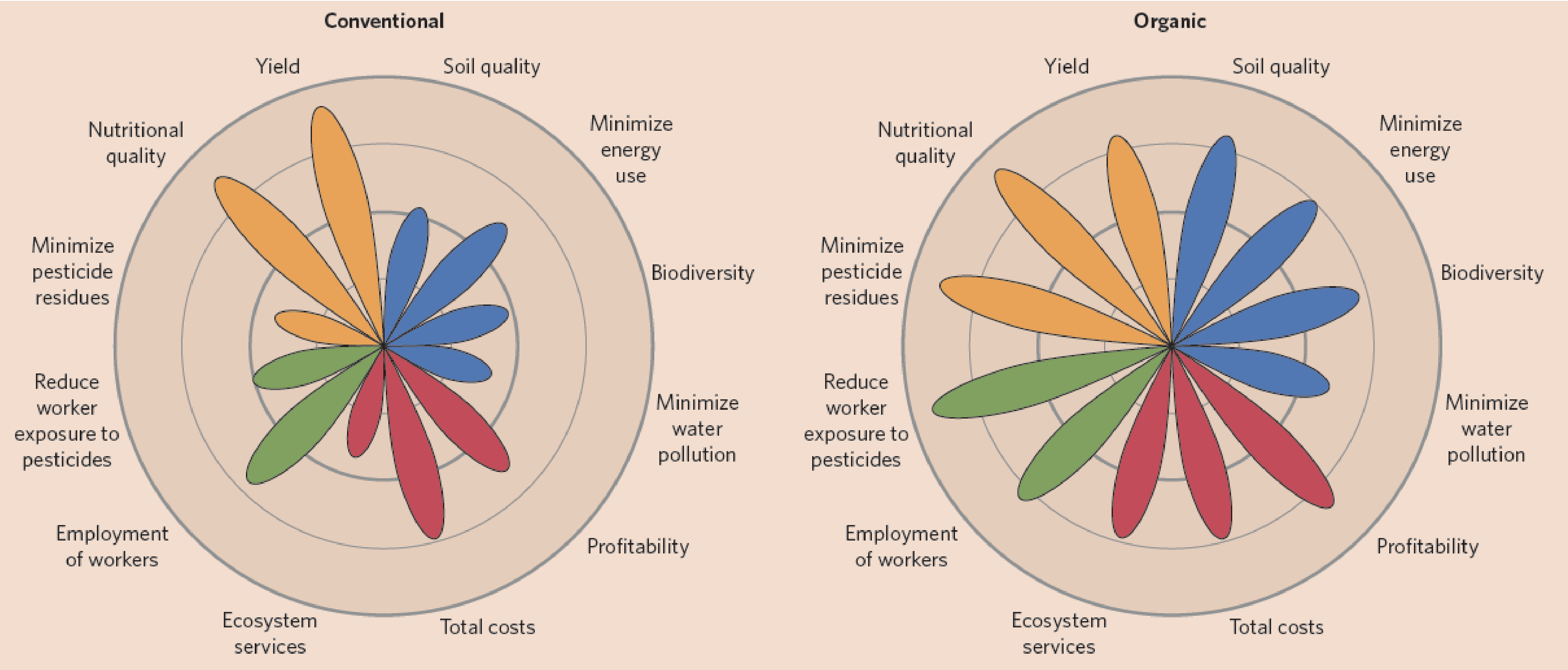

Figure 2. Assessment of conventional and organic farming in the four major areas of sustainability: orange for production, blue for environment, red for economic, and green for wellbeing. Lengths of the petals indicate the level of performance of specific sustainability metrics relative to the four circles indicating 25% to 100%. From Reganold & Wachter, 2016

Besides its positive influence on species abundance, richness, and diversity, organic farming may influence the breeding phenology and reproductive output of farmland birds. For instance, yellowhammer *Emberiza citrinella* initiated earlier first clutches and attempted second breeding on organic farms while having later first clutches and lacking double brood in intensive farms, probably linked to more abundant food supplies in organic farms (Bradbury et al., 2000). In skylarks *Alauda arvensis*, greater breeding density and success were found in organic compared to conventional fields, reflecting more invertebrate resources in organic fields that would otherwise be reduced by poorer vegetation structure and higher pesticide input (Wilson et al., 1997). Organic farming has, therefore, been proposed as a way of counteracting the global loss of biodiversity, at least under temperate latitudes (Stein-Bachinger et al., 2021) although this production system only covers 1% of global agricultural land (Reganold & Wachter, 2016). The only mandatory practice differentiating conventional and organic farming is the use of synthetic inputs, thus multiple studies emphasised the role of pesticide contamination in the disparities in biodiversity observed between conventional and organic farms. In European topsoils, organic farming fields had significantly fewer pesticide residues compared to conventional soils (Geissen et al., 2021), further supporting the role of pesticides in the contrasted biodiversity levels between conventional and organic farming.

II. Pesticides

1. Definitions, history, aims and classifications

Pesticides are used to kill, control or prevent crops against pests, weeds and vectors of diseases that may be harmful to the production, processing, storage, transport, or marketing of foods and other agricultural commodities (Khan & Rahman, 2017; EFSA, 2024). Chemicals to control pests and weeds first appeared with sulphur compounds used as insecticides 4,500 years ago (Bernardes et al., 2015; Zimdahl, 2015), but it is the discovery in the 1940s of dichlorodiphenyltrichloroethane (DDT) to kill vectors of malaria and typhus (Casida & Quistad, 1998), that paved the way for the development of synthetic pesticides in agriculture (Tudi et al., 2021). However, due to its ecological and sanitary consequences, persistence and bioaccumulation, DDT and most of its chlorinated derivatives (such as aldrin, dieldrin, endrin, chlordane) were banned in 2001 by the Stockholm Convention on Persistent Organic Pollutants (POPs). After their appearance, synthetic pesticides have undergone a large diversification, and their global production has soared, increasing from 0.2 million tons in the 1950s to more than 5 million tons by 2000 (Abubakar et al., 2020; Tudi et al., 2021).

Pesticides are often called phytopharmaceutical products or plant protection products (both referred to by the acronym PPPs), although the term pesticide also include other substances aiming to control pathogens and vectors of diseases and which are not intended for use on plants (e.g., organophosphates and synthetic pyrethroids against mosquitoes in malaria control; Dorta et al., 1993; EFSA, 2024). According to their intended use (agriculture, veterinary or medical for instance), pesticides do not rely on the same legislation and are thus not following the same ecotoxicity tests and approval process. Moreover, the term pesticide often refers to both the active substance (i.e., having the biocidal action) and the formulation of a PPP containing at least one active substance in a mixture with co-formulants or inert ingredients. The active substances present within a PPP might be synthetic chemicals, plant extract, pheromones or microorganisms (including viruses) and are generally categorised after their target (EFSA, 2024). The types of active substances include thus herbicides, fungicides, insecticides, acaricides, and rodenticides (EFSA, 2024; European Commission, 2024). Synergists are a type of co-formulant that enhance the action of the active substance(s), and thus, they are sometimes considered active ingredients, although they are not supposed to have biocidal properties (Abubakar et al., 2020; NPIC, 2024). Many other co-formulants might be present in PPPs, such as safeners that improve herbicide selectivity towards weeds rather than crop plants; solvents, adjuvants and carriers that enhance the dissolution, spreading, delivery and penetration of active ingredients in plant's leaf surface; or preservatives to extend the shelf life of products and prevent pesticide degradation due to exposure to sunlight (EPA, 2024b; European Commission, 2024; NPIC, 2024). In the present manuscript, the term "pesticide" will refer to the active substances present in a mixture while PPPs will refer to a formulation.

Besides their target-based classification, pesticides are divided into two chemical classes: inorganic (such as copper or sulphur) and organic pesticides. Pesticides within the organic class might be either natural (essential oil for instance) or synthetic (produced by chemical synthesis; Abubakar et al., 2020). Pesticides can also be classified according to their chemical structure (organochlorine or neonicotinoids for instance), modes of entry (systemic or non-systemic), functional group (notably repellents, chemosterilants, growth regulators), modes of action (acetylcholine agonists or protein synthesis inhibitors for instance), and toxicity (Bernardes et al., 2015; Deadman, 2017; Yadav & Devi, 2017; Abubakar et al., 2020; Tudi et al., 2021; see **TABLE A1** in Appendices). Nonetheless, some pesticides have unknown or unspecific modes of action (FRAC, 2024; HRAC, 2024; IRAC, 2024) and the production of wide-spectrum pesticides instead of pest-specific substances is increasing, raising concerns about their potential effects on non-target organisms. Besides, although pesticides with the same chemical structure are expected to present similar physicochemical properties and mechanisms of toxicity, it is not always the case and may depend on the organism studied. For instance, based on the oral and dermal LD50 (which is the median lethal dose that produces death in 50% of exposed animals), while all clothianidin, imidacloprid, thiacloprid and thiamethoxam neonicotinoids are of high toxicity to bees, only imidacloprid and thiacloprid are also considered highly toxic to birds (Lewis et al., 2016). Moreover, pesticides from different classes may have similar toxic effects (Bernardes et al., 2015). According to the World Health Organization (WHO, 2019), pesticides could be classified according to their toxicity using Acute Toxicity Hazard Categories based on the oral and dermal LD50 for rats (Yadav & Devi, 2017). This classification includes extremely (parathion) to slightly (malathion) hazardous classes and a last class of pesticides unlikely to present acute hazard (carbetamide) (Yadav & Devi, 2017; WHO, 2019). Nonetheless, all these toxicity-based classifications are only considering the acute toxicity to non-target model species, without accounting for chronic exposure and sublethal effects of wild organisms (see section "*II.4. Effects on non-target organisms*").

2. Legal framework of pesticide approval

a. Global disparities

Globally, for pesticides to be authorized in the market, the manufacturer must perform a suite of tests to ensure security to the environment, non-target species, food products, users and consumers and submit their application for approval to the responsible organization. However, it is up to each national authority to set its laws and regulations concerning pesticide production, sales and applications, leading to heterogeneous approval processes worldwide. Because countries have different requirements, guidelines, and legal limits, pesticide legislation varies greatly, particularly between developed and developing countries, which lack the resources and expertise to adequately implement and enforce legislation (Handford et al., 2015). The Food and Agricultural Organization (FAO), in collaboration with other interested groups, developed the *International Code of Conduct on Distribution and Use of Pesticides* in 1985 (updated in 2014) that aimed to harmonise regulation among pesticide exporting and importing countries (FAO & WHO, 2014), and regional organizations synchronizing existing laws of countries with similar legislation into a single regional framework appeared (Islam et al., 2017). However, there is still a lack of coordination in the regulatory processes among regional organizations and disparities remain. This heterogeneity in the legislative framework of pesticide approval results in certain substances approved for outdoor use in some countries being prohibited in others (Donley, 2019).

b. European pesticide regulation

i. Overview of the approval procedure

The European Food Safety Authority (EFSA) is responsible for the approval of pesticides in the European Union. The EFSA regulatory process is one of the most rigorous as the Regulation (EC) No 1107/2009 on the placing of pesticides on the market in Europe triggered the development of more accurate exposure modelling tools and risk-evaluation procedures (Handford et al., 2015; Geissen et al., 2021). In the EFSA procedure, new active substances must be reviewed by a "rapporteur" Member State through the application provided by the applicant. This document contains scientific information (including a literature review) and studies on the physical and chemical properties of the substance, its toxicology, its fate and behaviour within the environment and its effects on non-target species, including birds and mammals, aquatic organisms, arthropods, soil meso- and macrofauna and higher plants (Annex II and III of the Directive 91/414/EEC still applicable for Regulation (EC) 1107/2009). The EFSA provides guidance documents and technical guidelines for the information provided in the dossier to be considered valid[1]. For instance, to assess the fate and behaviour of pesticides within the environment, a series of laboratory and field tests is required in soil, water and air compartments, including aerobic and anaerobic transformation, rate of degradation, adsorption and desorption, column and field leaching, mineralization, photolysis etc. that all together allow to verify if the substance is considered a POP or not. For a substance to be approved (Regulation (EC) 1107/2009), it should not include all three points that characterize a POP, that is first, a half-life, also called DT50 (i.e., the time needed to obtain a degradation of 50% of the applied dose of pesticide) greater than 6 months in soil and sediments and 2 months in water, second, a high potential of propagation (i.e., detected over long distances) and lastly, a high bioaccumulation factor and/or toxicity to non-target species. Then, the report made by the "rapporteur" Member State is reviewed by all Member States and the EFSA to conclude the approval or not of the substance, which is presented to the European Commission (EC) that ultimately decides to approve it or not. Each Member State is free to implement its approval process for PPPs or other commercial products that contain the substance authorised by the EC. In France, the Agence nationale de sécurité sanitaire de l'alimentation, de

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[1] https://webgate.ec.europa.eu/dyna2/pgd/sections/283-A accessed on September 2024

l'environnement et du travail (ANSES) is responsible for the authorisation to place a PPP on the market or not.

In Europe, 1483 active substances are registered in the EU Pesticides Database[2], of which 425 are currently approved, 71 are pending (i.e., dossier considered valid, but the approval is still pending) and 20 are not yet assessed at the EU level (these include notably substances not considered a PPP under Dir. 91/414/EEC, such as safeners and synergists). In France, the ANSES e-phy database[3] currently registers 2875 PPPs authorised for professional purposes, while a hundred are authorised for domestic use, among which 1259 are fungicides, 1067 herbicides and 311 insecticides.

ii. Risk assessment for terrestrial organisms

For each ecotoxicological study on non-target organisms, a risk assessment from laboratory standard toxicity tests is required (following the standard protocols of the Organisation for Economic Cooperation and Development, OECD) and commonly uses quotients that combine exposure and effect to characterise the risk depending on the organism assessed. For instance, Toxicity to Exposure Ratio (TER) values for terrestrial vertebrates, earthworms or Hazard Quotients (HQ) values for bees (EU Guidance Document on Terrestrial Ecotoxicology - SANCO/10329/2002[4]). The TER corresponds to the measured ecotoxicological endpoint (of acute or sublethal effect) divided by the exposure and reflects thus higher a risk when lower. The risk assessment for birds and mammals is based on a three-tiered approach (EFSA, 2009). First, a "screening step" is realised to identify the substances that produce no detectable effects in a "worst-case" exposure scenario and that are thus considered of low-risk and not further included in the process. Substances that fail the "screening step" are tested in the first-tier risk assessment that uses more realistic exposure estimates to assess acute and reproductive risks. The first-tier generally uses the acute oral LD50 which is the lethal dose at which 50% of the tested group dies, as an endpoint to assess acute toxicity. The reproductive risk assessment is necessary unless the exposure is proved to not occur during the breeding season. To assess the reproductive toxicity on breeders fed with the substance, the no-observed-adverse-effect level (NOAEL) or no-observed-adverse-effect (NOEC) are generally determined as an endpoint although their relevance has been repeatedly discussed (Mineau, 2005; Moreau et al., 2022a). Substances that do not comply with the threshold limits of the first-tier risk assessment are refined in a higher-tier risk assessment (e.g. TER < 10 for acute risks and/or < 5 for reproductive risks). This step uses case-by-case approaches supposed to demonstrate if the substance produces "unacceptable impacts" under field conditions. Because no standardised approach exists for higher-tier assessments, one or more options are possible: field studies, population modelling, seed/granule availability, refined bioaccumulation modelling, phase-specific reproductive assessment, etc. (EFSA, 2009, 2023).

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[2] https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances accessed on September 2024

[3] https://ephy.anses.fr/resultats_recherche/ppp?search_api_aggregation_3=&sort_by=search_api_aggregation_4&sort_order=ASC&f[0]=field_intrant%253Afield_etat_produit%3A10 accessed on September 2024

[4] https://food.ec.europa.eu/system/files/2016-10/pesticides_ppp_app-proc_guide_ecotox_terrestrial.pdf accessed on September 2024

c. Regulatory flaws

Several limitations to these toxicity tests have been highlighted in field studies (Mineau, 2005; Lopez-Antia et al., 2016). First, risk assessment is based on TER considering predicted environmental concentrations of a single residue in the soil after its application following recommended schemes. Therefore, this does not account for different soils and climatic conditions that may lead to marked differences in degradation rates, or for the historical contamination due to banned substances, which may result in an underestimation of the risk to soil organisms notably (Kah et al., 2007; Geissen et al., 2021). Besides, tests are performed on standard species that are quail species (Japanese quail, *Coturnix coturnix japonica* or bobwhite quail, *Colinus virginianus*) or mallard duck *Anas platyrhynchos* (EFSA, 2009, 2023). The reported estimates of the quantity (dose or concentration), frequency, and duration of exposure to the substance while considering "all relevant factors" fail to reflect realistic conditions. Indeed, as no mandatory assessment of specific factors is given, some substances lack information about their risk depending on the species' biology, diet, or time spent in treated areas for instance (Morrissey et al., 2023). The endpoints used to assess acute and chronic effects are different concentrations, levels or doses (NOEC/ NOAEL/ Lowest Observed Effect Concentration or Level (LOEC/LOEL) and Lowest Observed Adverse Effect Level (LOAEL)) that are directly derived from the experimental design and interpreted as the limit at which an active substance switches from safe to toxic, thus being misleading and neglecting biological variability (Laskowski, 1995). Moreover, all tests are performed in a single-substance manner, so that cocktail effects on non-target organisms are neglected (Moreau et al., 2022a). Therefore, for birds and mammals, the extrapolation of laboratory short-term toxicity in mono-species to field long-term toxicity on wild species is unrealistic. For instance, neonicotinoids were presumably of low toxicity to non-target vertebrates, with a NOAEL of imidacloprid for instance of 9.3 $mg.kg^{-1}bw.d^{-1}$ determined in *Colinus virginianus*. However, some red-legged partridges fed with low doses of 8.8 $mg.kg^{-1}bw.d^{-1}$ died within 3 days of exposure and those who survived to the 28 days of exposure showed sublethal effects including fewer eggs laid and delayed first egg laid (Lopez-Antia et al., 2015a) which may have ultimate consequences on the population's size. Besides failing to predict the doses causing sublethal effects in other bird species, this testing scheme fails to reflect long-term effects even in the tested species. Indeed, the accelerated declines of *Colinus virginianus* have been linked to the introduction of neonicotinoids and their widespread use in Texas croplands (Ertl et al., 2018). Therefore, the regulation of pesticides appears to be inadequate and might explain the environmental issues and unintended effects on non-target organisms observed nowadays (see the following sections). Future improvements in the ability to predict the long-term fate and effects of pesticides beyond what is known from regulatory tests are needed (Fenner et al., 2013; Morrissey et al., 2023).

3. Pesticides within the environment

There are different types of application of PPPs: spraying, coating around seeds, or as granular direct application. Spraying onto crops or weeds is the most common application method, although the treatment of seeds before their sowing is increasingly used for insecticides and fungicides application (Knowles, 2008). The timing, number and intervals of PPP application depend on crop's phenology, on the phenology of pathogens/weeds/pests and the duration of protection of the PPP, modulated by on-field weather (Räsänen et al., 2023; FAO, 2024).

Consequently, multiple PPPs are applied in fields either at the same time or at different time intervals across the year (Räsänen et al., 2023).

Once applied, pesticides enter target organisms but also the environment. Spray drift, volatilization, leaching and run-off contaminate the air and water bodies, transporting pesticides to non-treated areas (**Figure 3**; Tudi et al., 2021). Generally, during application less than 1% of PPPs applied reach the target pests and weeds (Bernardes et al., 2015). Spraying application method presents a high risk of transfer in the environment depending on the meteorological conditions (Knowles, 2008; Tudi et al., 2021). Indeed, wind speed and direction, air temperature and relative humidity are key factors influencing the spray droplet direction and evaporation, leading to off-target deposition of pesticides (Fishel & Ferrell, 2010). Although coated seeds are supposed to be of lower risk for the environment compared to spraying, events of massive environmental and biological contamination might occur notably in case of abrasion of dust from seeds coated with pesticides (Pistorius et al., 2009). Besides, soil characteristics (porosity, clay and organic content, sorption capacity, etc.) are as important as pesticide characteristics (chemical structure, volatility, solubility, stability, etc.) in processes of transfer in environmental compartments (Al-Mamun, 2017).

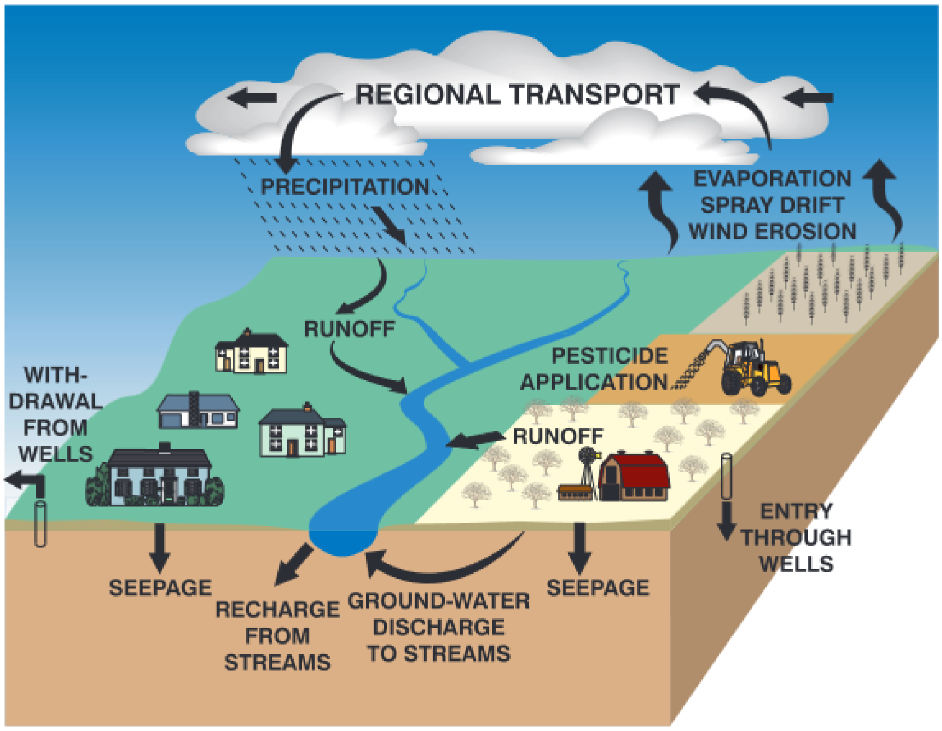

Figure 3. Transfer routes of pesticides in surface and ground waters. From Barbash & Resek, 1996.

Pesticides are supposed to be degraded in the environment through abiotic and biotic processes such as mineralisation, photolysis or oxidation, resulting in the formation of new chemicals (**Figure 4**). The soil's adsorption of pesticides increases the chance of degradation although the remaining by-products can accumulate in soils and be transferred to multiple environmental compartments (Fenner et al., 2013; Al-Mamun, 2017). Exposed target and non-target organisms also participate in pesticide degradation through metabolism or co-metabolism processes that result in the formation of metabolites excreted back to the environment (Van Eerd et al., 2003; Katagi & Ose, 2015; **Figure 4**). Phytoremediation is notably a strategy used to counteract soil pollution, through which plants and their associated microorganisms degrade, detoxify, and remove pesticides (Van Eerd et al., 2003). Metabolites and non-degraded pesticides can also be stored within organisms under bioavailable forms or not (in the liver or feathers/hairs, for instance) and might then bioaccumulate within the organism or biomagnify along the trophic chain depending on their properties (Smith et al., 2007; Katagi, 2010; Katagi & Fujisawa, 2021; Tison et al., 2024). Although pesticide transformation products are supposed to have a lower effect than the original chemical, some can have equal/higher toxicity or unintended effects (endocrine disruption or other chronic effects) (Fenner et al., 2013; Al-Mamun, 2017). Among the 21 transformation products resulting from various degradation processes of four widely used parent pesticides, 50% induced stronger endocrine-disrupting effects than parent compounds *in vitro* and *in silico* (Ji et al., 2020). From *in vivo* tests, the chlorothalonil metabolite 4-hydroxychlorothalonil induced a 2.6-fold higher mortality rate than the parent compound on zebrafish (*Danio rerio*) embryos and acted as a thyroid receptor agonist and antagonist in gene assay while this was not observed for the parent compound (Zhang et al., 2016).

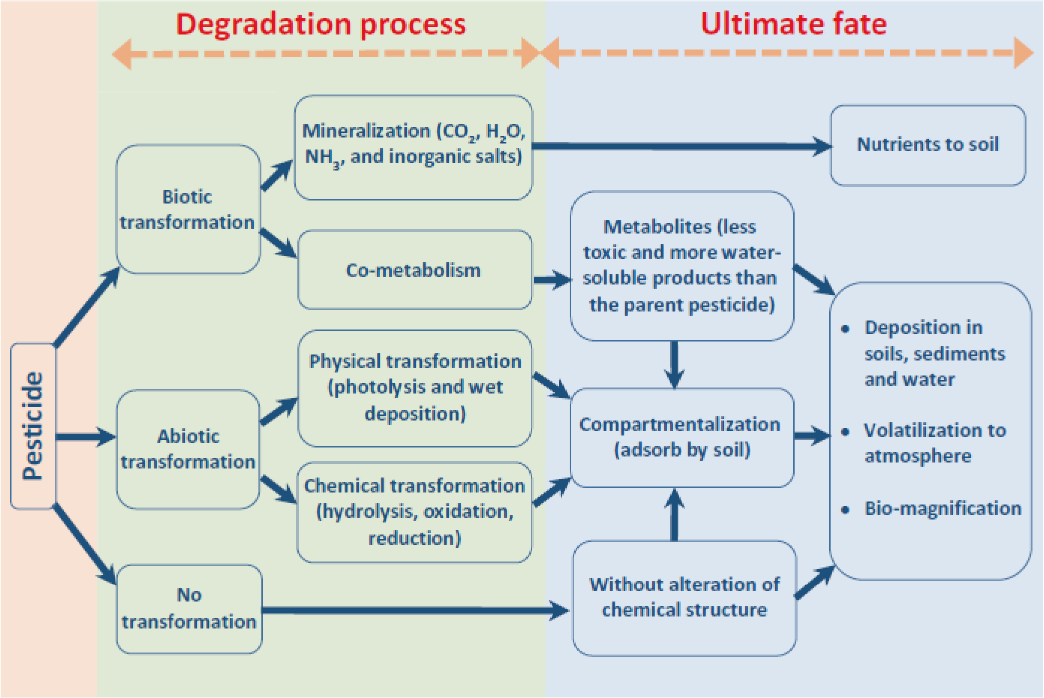

Figure 4. Fate of pesticides and their degraded compounds in the environment. From Al-Mamun, 2017.

The half-life (DT50) of pesticides is supposed to last from few days to weeks (depending on the intended period of use) and it is now acknowledged that laboratory studies generally overestimate the degradation of pesticides (Fenner et al., 2013; Turco & Kladivko, 2018) even though some compounds show faster degradation under field conditions (Tsochatzis et al., 2013). Pesticide degradation rate depends on its chemical structure (larger molecules and aliphatic/aromatic contents have a slower degradation rate), field characteristics (soil's microbial community or porosity), and environmental conditions (temperatures, pH, nutrients, sunshine or rainfall) (Kah et al., 2007; Al-Mamun, 2017). Thus, depending on the substance and the conditions, the persistence within the environment may last hours to weeks, even decades in some cases. For instance, in French soil samples, six substances were detected more than three years after their last application reported by farmers and far exceeded their theoretical DT50 and even their DT90 (90% of the applied dose degraded) (Froger et al., 2023). In other countries in Europe, similar patterns are reported with the detection of substances in organic fields, sometimes untreated for 20 years, and in non-treated areas such as forests and permanent grasslands (Riedo et al., 2021; Geissen et al., 2021). Fog water and air samples also allow to assess the persistence of pesticides as some substances detected at significant levels in urban sites were banned for decades, which also highlights their long-range transport from rural to urban sites and their potential transfer among environmental compartments (Zaller et al., 2022; Khoury et al., 2024).

Consequently, numerous studies detected currently used or legacy pesticides, by-products and metabolites in abiotic and biotic environmental compartments worldwide (Al-Mamun, 2017; Zubrod et al., 2019; UNEP, 2022). This global contamination, especially in agroecosystems pose serious questions concerning their impacts to non-target organisms, including humans.

4. Effects on non-target organisms

Pesticides entering non-target species might have both indirect (i.e., ecological interactions) and direct effects (i.e., toxicity) to organisms, resulting in wild populations' decline (**Figure 5**).

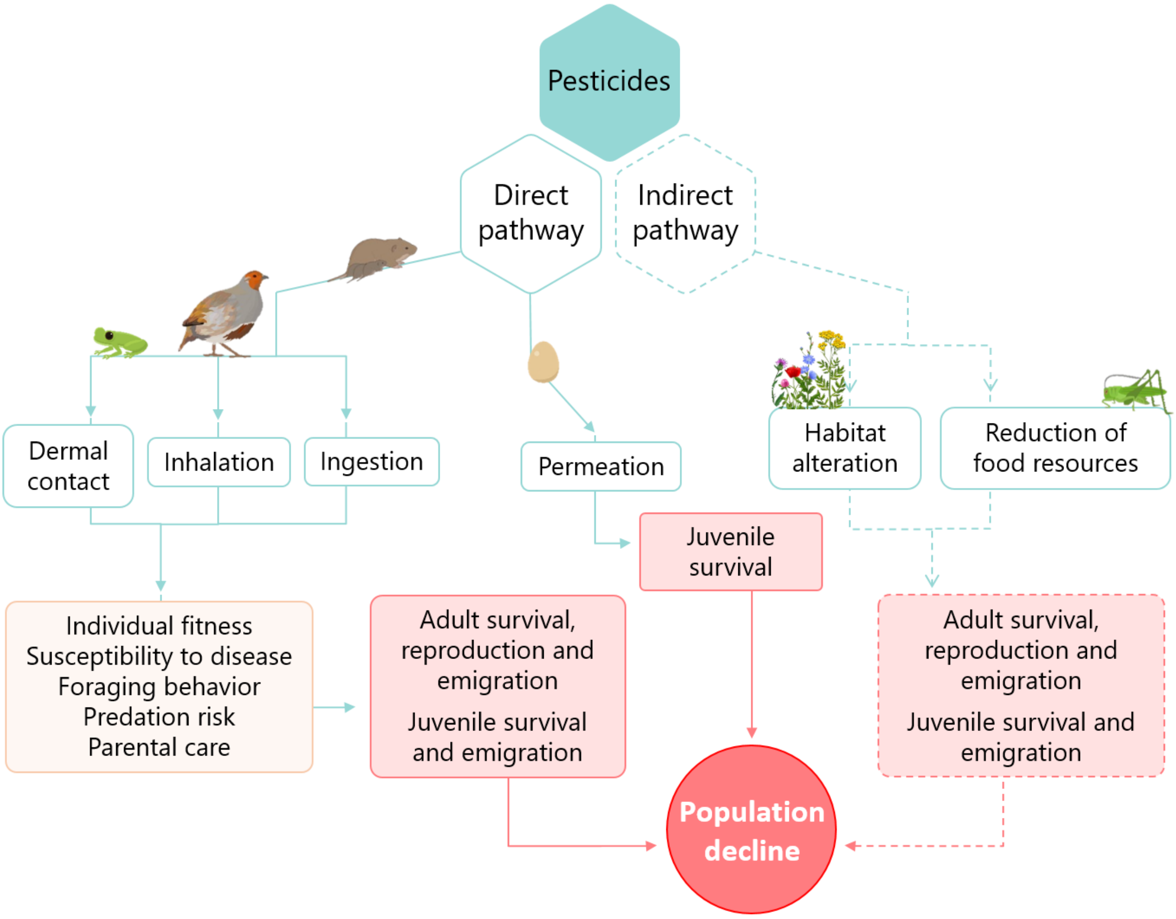

Figure 5. Direct and indirect pathways of pesticide effects on non-target organisms that may cause population declines in terrestrial vertebrates. Inspired by UNEP, 2022.

a. *Indirect effects*

Indirect effects of pesticides include the reduction of habitats and food resources available for many species (**Figure 5**; Stanton et al., 2018; Fritsch et al., 2024). Worldwide, insects have suffered severe declines due to pesticide use, both directly because of insecticide toxicity (see section "*II.4.b. Direct effects*") and indirectly through the reduction of plant biodiversity (Sánchez-Bayo & Wyckhuys, 2019). Herbicide use reduces the abundance and diversity of wild plants that are essential as shelters for some invertebrates to complete their life cycle or as food resources for herbivorous species (Sánchez-Bayo, 2021). Consequently, beneficial organisms (parasitoids and predators of pests, pollinators, etc.) are scarcer in pesticide-treated plots than in untreated plots (Sánchez-Bayo, 2021).

In vertebrates, pesticides may also cause indirect effects through the reduction of food resources (weed seeds and invertebrate prey) and/or natural habitats. Populations of two lizard species have been affected by fipronil (insecticide) spraying as it reduced the populations of their main prey (Peveling et al., 2003). Neonicotinoids can also indirectly affect bat species as their diet relies on aquatic invertebrate hatches, which are drastically impacted by this class of insecticides (Sánchez-Bayo et al., 2016; Mineau & Callaghan, 2018). The lower abundance of invertebrate prey linked to neonicotinoid applications induces a reduction of the growth of aquatic vertebrates such as the medaka fish *Oryzias latipes* (Hayasaka et al., 2012). Besides, herbicides can alter aquatic trophic chains through their toxic effect on phytoplankton, causing indirect effects on zooplankton and macroinvertebrates (Hasenbein et al., 2017; Sánchez-Bayo, 2021).

The indirect effects of pesticides on non-target organisms also include disruption of pathogens or parasites interactions with their hosts. Cases of beneficial effects include notably Cape ground squirrels *Xerus inauris* exposed to fipronil that benefit from ectoparasite removal and have higher breeding success (Hillegass et al., 2010). In aquatic vertebrates, fungal infections are reduced due to the toxicity of fungicides to pathogens (Zubrod et al., 2019). However, many pesticides may have direct sublethal effects on the immune system of host species, thus their net effect on the prevalence of diseases/parasites will depend on their toxicity to both involved species (Mason et al., 2013; Zubrod et al., 2019).

b. *Direct effects*

Contamination with large amounts of pesticides may be lethal (short-term effect) while repeated (i.e., in case of ≤28 days studies; OECD, 1993), subchronic (<10% of the animal's lifespan) or chronic (>10% of the animal's lifespan) exposure to small amounts of pesticides may induce sublethal effects (delayed long-term effects).

i. Lethal effects

Direct lethal effects of pesticides result from the toxicity of pesticides that can lead to the death of organisms after acute exposure through dermal contact, inhalation, ingestion or permeation (**Figure 5**). In humans, over 150000 deaths from pesticide acute exposure occur every year, most from self-poisoning by ingestion (Eddleston, 2020), although the fatality rate varies depending on the substance, age and sex of the individual (Dawson et al., 2010).

Many beneficial organisms are declining due to the acute toxicity of pesticides applied in fields. Soil invertebrates are notably essential for soil structure and fertility and are strongly affected by insecticides such as carbamates, which are very toxic to earthworms (Al-Mamun, 2017). Although most herbicides are not acutely toxic to soil organisms, some substances such as atrazine might reduce the abundance of wireworms and springtails (Sánchez-Bayo, 2021). Predators such as coccinellids, spiders and mites participating in the biological control of pests suffer higher mortality than pest species due to their higher sensitivity to some insecticides and herbicides (Al-Mamun, 2017; Sánchez-Bayo, 2021). Neonicotinoid insecticides, for instance, are particularly toxic to natural enemies and pollinators present in fields (Kumar et al., 2012; Al-Mamun, 2017). The synergism among pesticide substances has notably been found to pose a high risk of toxicity to bees (Sánchez-Bayo & Goka, 2014).

Vertebrate wild species are subject to the direct lethal effects of pesticides. Acute poisonings of wild birds with legacy anticoagulant rodenticides, organochlorine insecticides, organophosphate and carbamate pesticides have been heavily reported, sometimes resulting in severe population declines (Newton & Wyllie, 1992; Mineau et al., 1999; Stone et al., 1999). Currently-used pesticides such as the neonicotinoid imidacloprid or the fungicide pyraclostrobin have been found to produce mortality events, even at low doses, in granivorous birds and amphibians respectively (Fritsch et al., 2024; Zubrod et al., 2019). In bat species, organochlorine insecticides have been associated with multiple mortality events (O'Shea et al., 2016). The age or life stage of organisms, the concentrations and the time of exposure are important in the susceptibility and risk of lethal effects of pesticides. For instance, amphibian eggs and tadpoles exposed to environmentally relevant concentrations of pyrethroids insecticides might survive, and during development, the abnormalities in morphology and behaviour would further lead to mortality (Greulich & Pfugmacher, 2003). Thus, low concentrations considered sublethal during short-term exposure may be lethal if the exposure is chronic (Bridges, 2000).

ii. Sublethal effects

Direct sublethal effects result from exposure to low doses of pesticides that induce long-term consequences on the organisms, ultimately affecting their survival, reproduction and/or emigration (**Figure 5**). For instance, neurologic, pulmonary, reproductive, developmental and carcinogenic effects can be found in humans exposed chronically or sub-chronically to pesticides (Debnath & Khan, 2017).

Pesticides from multiple chemical families (organophosphates, organochlorines, carbamates) including the compounds glyphosate and cypermethrin studied alone or in mixtures have been found to affect earthworm growth and reproduction (see Yasmin & D'Souza, 2010 for a review) and to depress the immune system and thus increase diseases/parasites in both invertebrate and vertebrate populations exposed (Christin et al., 2003; Mason et al., 2013). For instance, one of the suggested mechanisms of bee colony collapse involves the interaction of pathogens with imidacloprid, as the pathogen's growth within bees would be higher in colonies exposed to this neonicotinoid (Pettis et al., 2012). Neonicotinoids (alone or in combination with other neurotoxic insecticides) have been largely studied for their sublethal effects on the foraging, learning and memory abilities of bees, decreasing the efficiency of pollen collecting and eventually leading to colony collapse (Gill et al., 2012; Williamson & Wright, 2013). Glyphosate-based herbicides also affect the behaviour of invertebrate natural enemies resulting in lower biological control (Al-Mamun, 2017).

In vertebrates, neurotoxic compounds (carbamate, organochlorine, organophosphate and neonicotinoid insecticides) may also induce altered feeding activity and movements and reduced antipredator behaviours (amphibians, Sievers et al., 2019; lizards and bats, Fritsch et al., 2024). Besides, fungicides acting as demethylation inhibitors are of concern because of their endocrine-disrupting effects on amphibians and fish, altering gonadal development, sex ratios and fecundity (Zubrod et al., 2019). Metabolic disturbances and alterations of thermoregulation have been reported in small mammals exposed to organophosphates (Fritsch et al., 2024). Birds are also subject to numerous sublethal effects, including endocrine disruptions, biochemical alterations and behavioural changes that alter their reproduction and survival (see the detailed section "*II.3. Sublethal effects of pesticides on farmland birds*").

iii. Global effects

Overall, the indirect effects of pesticides on vertebrate wildlife are at least as important as direct toxic effects although causal links are often difficult to assert (Gibbons et al., 2015; Fritsch et al., 2024). Direct lethal effects of pesticides are more striking concerning invertebrates and were reported in vertebrates mainly for legacy persistent pesticides, although incidents of vertebrate mortality linked to currently used pesticides have been reported recently (Botha et al., 2018; Hong et al., 2018; Coeurdassier et al., 2019). Nonetheless, accidental mortality events alone cannot be considered the principal cause of farmland birds' decline (Fritsch et al., 2024). Direct sublethal effects are undeniably contributing to populations' decline, however, these are complex to spot and study as they can be delayed (or carryover) effects, and that trade-off within organisms might hide their consequences (Zgirsky et al., 2021; Moreau et al., 2022a). Nonetheless, the combination of both indirect and direct effects is generally responsible for the adverse effects of pesticides on terrestrial vertebrates (Fritsch et al., 2024).

c. Birds decline worldwide and the role of pesticides

Birds are well-known organisms, notably due to their long-term monitoring in numerous countries. These monitorings allowed the documentation of declining trends of bird populations from several taxonomic groups since the 1950s (Rosenberg et al., 2019) with farmland birds showing the steepest decline in Europe and North America (Inger et al., 2015; Heldbjerg et al., 2018; Reif and Hanzelka, 2020; Rigal et al., 2023). Contrary to generalist species, farmland birds are less flexible in the resources they exploit (i.e., within agricultural areas), which makes them vulnerable to any change within their narrow ecological range. The grey partridge *Perdix perdix*, a flagship species in Europe, displays declining population trends that have been associated with the reduction of invertebrates to feed chicks due to pesticide use, resulting in lower reproductive success (Rands, 1985). The insecticide-driven reduction of food resources has also been associated with reductions in the reproductive success of at least four passerine bird species in the UK although it does not explain alone the population's decline (Gibbons et al., 2015). Over-winter survival of these species is notably compromised by the reduction of food resources due to herbicide use that causes reductions of both weed seeds and host plants for invertebrate prey (Robinson & Sutherland, 1999; Burn, 2000). Because pesticide use seemingly affects avian populations, the monitoring of these species may provide early warning signals of environmental changes, notably of the consequences of pesticide use on agroecosystem health (Baos & Blas, 2008; Mitra et al., 2011; Moreau et al., 2022a). Moreover, birds have been used for decades for the biomonitoring of pollutants because they occupy multiple positions in the trophic chains and are relatively easy to sample through non-lethal methods (Becker et al., 1994; Becker, 2003). Therefore, these are valuable characteristics to consider farmland birds as biosentinels of pesticide effects in agroecosystems.

III. Farmland birds as biosentinel species of pesticide effects

1. Linking pesticide use to farmland birds' decline

Evidence of the role of intensive farming practices in farmland birds decline accumulated worldwide (Chamberlain et al., 2000; Geiger et al., 2010; Stanton et al., 2018) although the main contributor amongst each component (mechanisation, land-use change, synthetic inputs) has been debated. In Europe, the massive use of synthetic fertilisers and pesticides has been pointed out as the main driver of farmland bird decline (Rigal et al., 2023). However, the contribution of pesticides to bird decline has been mainly assessed indirectly and the underlying mechanisms (indirect vs. direct effects) remain generally unknown. Raptors consuming prey contaminated with organochlorine insecticides were among the first investigations directly linking the sublethal effects of pesticides to birds' decline. For instance, in Britain, the shell-thickness of Sparrowhawks, *Accipiter nisus,* eggs became thinner after 1947, coinciding with the widespread use of DDT (Newton, 1995). The restriction of use since the 1970s and subsequent ban led to the progressive recovery of eggshell thickness and populations (Newton, 1995). Other similar effects and trends were reported for other raptor species and in other countries, pointing out the global adverse effects of this class of pesticides (Ratcliffe, 1967; Newton & Wyllie, 1992; Oli et al., 2023 and references therein). These effects first observed from field studies were confirmed by experimental studies (Cooke, 1973). Because of its toxicity, persistence, long-range transport and bioaccumulation, DDT, along with other organochlorine insecticides have been included in the list of POPs and banned for use (except to control vector-borne diseases). Organophosphorus and carbamate insecticides that were subsequently developed were supposed to have lower persistence in the environment and lower risks of toxicity (Abubakar et al., 2020). Nonetheless, further studies emphasised the adverse effects of organophosphates and carbamates notably on birds' orientation abilities, immune system, and reproductive functions (Mitra et al., 2011). Apart from legacy organochlorines, organophosphates and carbamates, more recent studies investigated the toxicity of currently used pesticides, such as neonicotinoid insecticides or triazole fungicides, to farmland birds (Moreau et al., 2022a). The findings of these works are valuable to public policies as scientific evidence of adverse effects can lead to the ban of some pesticides locally (neonicotinoids in France, for instance; Judgment of the Court (First Chamber) of 19 January 2023). For instance, the reduction in northern bobwhites' abundance has been linked to their increased exposure to neonicotinoid insecticides (Ertl et al., 2018). However, contrary to the example of DDT, no study directly assessed the consequences of the sublethal effects of currently used pesticides on farmland birds' population trend, probably due to the difficulty of considering the environmental complexity of pesticide cocktail effects *in natura* and of monitoring wild species in the long-term.

2. Farmland bird exposure to pesticides

The exposure pathways of wild birds to pollutants are well documented and include ingestion, dermal contact, inhalation or maternal transfer (Smith et al., 2007). Wild farmland birds may be exposed to pesticides through the ingestion of contaminated food and water, and contact with contaminated soil, rain, and vegetation (**Figure 5**; Smith et al., 2007; Mineau, 2011). In case of contact exposure, birds may absorb substances by dermal contact and ingestion after feather preening, for instance (**Figure 5**; Smith et al., 2007). Another important pathway, although often neglected, is the direct inhalation of pesticide particles (**Figure 5**; Driver et al., 1991). Eggs can be contaminated through direct contact with pesticides (sprayed or persisting on the vegetation/soil and adults' feathers) that enter the eggshell by permeation (**Figure 5**; Bro et al., 2016; Ortiz-Santaliestra et al., 2020). Besides, a maternal transfer of some compounds might occur (e.g., Bellot et al., 2022a), contaminating eggs and chicks that were not directly exposed (**Figure 5**). The oral ingestion is the main studied exposure route in risk assessment studies, and despite the implementation of field exposure scenarios in some experiments, the contamination pathways under field conditions are much more complex (Mineau et al., 1990; Smith et al., 2007; Mineau, 2011). Indeed, as stated previously, pesticide application methods are diverse (coated seeds or spraying for instance) and may favour one or another route of exposure (e.g., pesticides around coated seeds would enter mainly by the ingestion pathway). Besides, pesticide applications can occur multiple times across the year, multiplying the timing of exposure, and resulting in contamination with cocktails of pesticides at any time (Bro et al., 2015).

Primary poisoning differs from secondary poisoning in that primary poisoning is the direct contact with pesticides through the preening or consumption of pesticides applied on seeds or meat baits, while secondary poisoning occurs from the consumption of contaminated organisms (prey or seeds from treated plants) (Mendenhall & Pank, 1980; Berny & Gaillet, 2008). Secondary poisoning presumably exposes individuals to lower concentrations than primary poisoning. However, depending on the affinity of pesticides with biological matrices, some compounds are more or less subject to bioaccumulation in tissues and/or biomagnification along the trophic chain, sometimes resulting in lethal effects (Krone et al., 2017; see Tison et al., 2024 for a review).

3. Sublethal effects of pesticides on farmland birds

The sublethal effects of pesticides can be assessed on several aspects of farmland birds' health (morphological, physiological and behavioural traits) and reproduction (**Figure 6**), which may ultimately affect population trends (**Figure 5**).

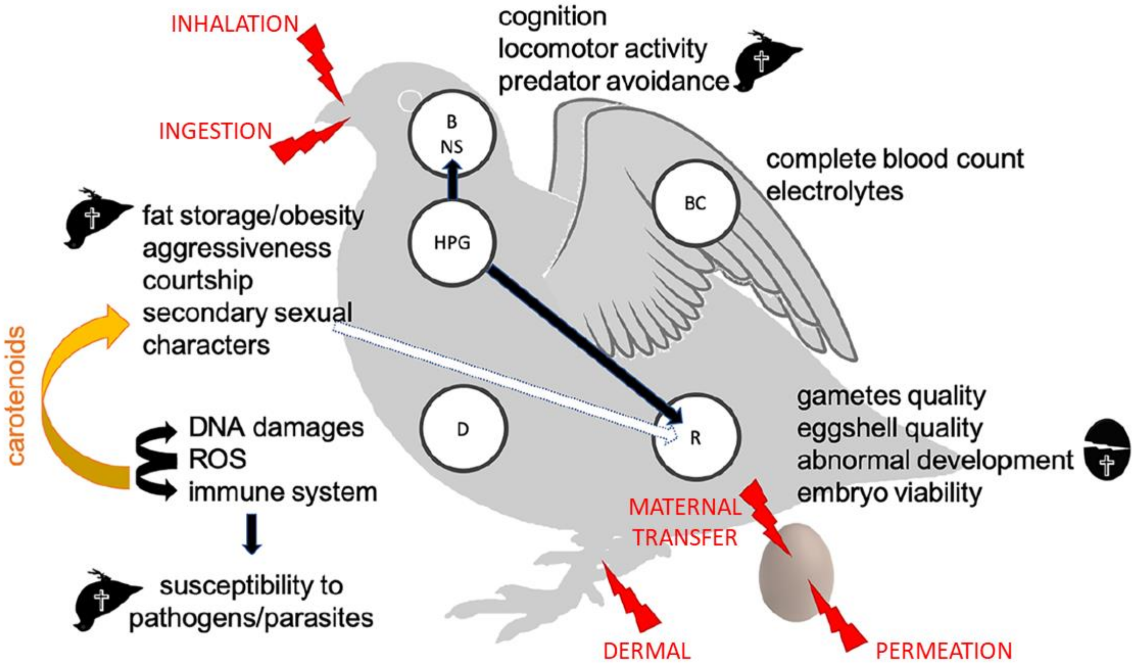

Figure 6. Schematic representation of birds' exposure pathways (red lightning bolts) to pesticides and their sublethal effects. Major physiological functions or systems are represented in white circles. B: brain, NS: nervous system, HPG: hypothalamic-pituitary-gonadal axis, BC: homeostasis, D: detoxification, R: reproduction. Black arrows represent the direct effects of one function on another. The white dotted arrow indicates an indirect link (behaviour). Modified from Moreau et al., 2022a.

a. Blood chemistry

Altered biochemical parameters (such as glucose, calcium, cholesterol, magnesium, albumin, protein, triglyceride concentrations) and haematocrit after ingestion of currently used pesticides have been found in partridges (Lopez-Antia et al., 2013; 2015a; 2015b; 2018; 2021; Moreau et al., 2021; Fernández-Vizcaíno et al., 2020; **Figure 6**) and in cockerels *Gallus domesticus* (Hussain et al., 2019). Red-legged partridge chicks hatched from eggs sprayed with tebuconazole (triazole fungicide) or 2,4-D (phenoxycarboxylate herbicide) also show altered biochemical parameters (Ortiz-Santaliestra et al., 2020). These biochemical parameters are linked to major functions of the organism and are thus often associated with other life-history trait alterations, evidencing the adverse effects of pesticides on global organisms' health.

b. Endocrine disruptions resulting in morphological alterations

Alterations in the volume of thyroid glands, damage to thyroid follicles, lesions in the stroma and altered secretion of thyroid and pituitary hormones, after exposure to low doses of either mancozeb or imidacloprid have been reported (Pandey & Mohanty, 2015). Pesticides may have endocrine-disrupting effects through different pathways, notably the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic-pituitary-gonadal (HPG) axis and the hypothalamic–pituitary–thyroid (HPT) axis, therefore, they can alter multiple hormone-mediated processes in the organism. The HPA axis mainly controls bodily responses to stress through the secretion of glucocorticoids (corticosterone hormone in birds), the HPG axis coordinates reproduction through the secretion of steroid hormones, while the HPT axis regulates energy metabolism and development through the secretion of thyroid hormones, although the HPT axis also plays an important role in the endocrine regulation of reproduction (Mohanty, 2024). A chronic increase of corticosterone levels and/or thyroid hormones may result in fat store depletion, muscle loss, growth and immune system suppression notably (McNabb, 2007; Baos & Blas, 2008). For instance, reduced body condition was reported after ingestion of imidacloprid-treated seeds (Lopez-Antia et al., 2013; 2015a), thiram-treated seeds (Lopez-Antia et al., 2015a) or fipronil-treated seeds (Lopez-Antia et al., 2015b) in the red-legged partridge *Alectoris rufa*. In grey partridges, however, higher body conditions were reported only for females fed with 'conventional' grains (containing pesticide mixtures) compared to females fed organic (Moreau et al., 2021; **Figure 6**). Females house sparrow *Passer domesticus* exposed to tebuconazole through drinking water also had higher body condition than unexposed individuals (Bellot et al., 2022b). Sex-dependent endocrine disruption altering energy regulation and fat deposition may explain the observed sex-specific pesticide effects on body condition (Moreau et al., 2021; Bellot et al., 2022b). Besides, house sparrows exposed to tebuconazole had both an altered plumage structure and reduced levels of thyroxine, a thyroid hormone known to be involved in the moulting process of birds, suggesting a disrupting effect of tebuconazole on the HPT axis resulting in the altered feather quality (Bellot et al., 2023). Delayed development of body plumage and flight feathers was observed in Japanese quails *Coturnix japonica*, especially in females, which are fed on seeds treated with glyphosate-based herbicides although the underlying mechanisms were not discussed (Ruuskanen et al., 2020a). Taken together, these effects can have ultimate consequences for the survival of individuals. Indeed, too low body condition can further reduce general health (reduced energy reserves), too high body condition can increase wing loading and altered plumage can reduce flight ability, all of which can prevent wild birds from escaping predators (Swaddle et al., 1996; Lind et al., 2010; Van den Hout et al., 2010).

As previously mentioned, endocrine disruptions linked to pesticide exposure may alter growth in developing birds. For instance, high levels of DDT in plasma of Bonelli's eagle *Aquila fasciata* nestlings were associated with reduced body condition (Ortiz-Santaliestra et al., 2015). This is not limited to legacy organochlorine insecticides as nestlings of barn owls *Tyto Alba javanica* fed with prey contaminated with rodenticides had reduced growth performance and were smaller than nestlings fed with uncontaminated prey (Naim et al., 2010). Similarly, common kestrel *Falco tinnunculus* nestlings contaminated with rodenticides had lower body mass than uncontaminated nestlings (Martínez-Padilla et al., 2017). However, male sparrows exposed to acetamiprid displaying higher corticosterone levels in feathers did not display an altered body mass compared to unexposed individuals, suggesting that disruptions in glucocorticoid secretion do not necessarily result in body mass depletion (Humann-Guilleminot et al., 2024).

c. Oxidative stress and detoxification

Because pesticides increase the production of reactive oxygen species (ROS), lipid peroxidation and DNA damage, oxidative stress has generally been found to increase after exposure to low doses of clothianidin (Hoshi et al., 2014) or imidacloprid (Lopez-Antia et al., 2013; 2015a; Abu Zeid et al., 2019) (**Figure 6**). The detoxification function that prevents the accumulation of cytotoxic ROS in organisms may be impaired by pesticides and lead to important oxidative damage (Hoshi et al., 2014). Nonetheless, it is not a rule of thumb and depending on the substance and species tested, increased antioxidant defences, or no change in oxidative stress may also be found (thiram or difenoconazole, Lopez-Antia et al., 2013; fipronil, Lopez-Antia et al., 2015b; acetamiprid, Humann-Guilleminot et al., 2024). Carotenoids involved in antioxidant defences, are also involved in coloured ornaments. Therefore, the greater allocation of carotenoids to detoxification processes in the case of pro-oxidant effects of pesticides may induce a reduction in carotenoid-based colouration (Lopez-Antia et al., 2018). Besides, because these pigments are also involved in immunocompetence, and free radicals are important in immune system regulation, alterations in the oxidative status of birds may also alter their immune responses (Lopez-Antia et al., 2013).

d. Immune system and host-pathogen interactions

The immunotoxicity of pesticides has been evidenced for a few compounds and consequences diverge between decreased or over-activated immune responses, sometimes associated with body condition or hormonal alterations (Lopez-Antia et al., 2013; 2015b) and with potential consequences on host-pathogen dynamics (Moreau et al., 2021). For instance, in grey partridges fed with pesticide mixtures (conventional grains), both the immune system and the intestinal parasite load were higher than in grey partridges fed organic (Moreau et al., 2021). On the opposite, the acquired immune response in male red-legged partridges was lowered by the consumption of thiram or imidacloprid-treated seeds (Lopez-Antia et al., 2013). However, in domestic chickens, none of the immune functions assessed were affected by imidacloprid, potentially due to the lower doses ingested per day compared to red-legged partridges (Franzen-Klein et al., 2020). Any alteration in the immune system of organisms increases the susceptibility to diseases and compromises the resistance to pathogens, and therefore impairs survival.

e. Microbiome dysbiosis

A new body of evidence also points out the sublethal effects of pesticides on microbiomes and suggests that this may induce adverse effects on different health components (Crisol-Martínez et al., 2016; *ARTICLES 7 and 8 in Appendices*). In Japanese quails, ingestion of glyphosate-based herbicides led to a gut microbiome dysbiosis that may be associated with the observed reduction in testosterone secretion and antioxidant activity of the catalase enzyme (Ruuskanen et al., 2020b). Moreover, in males, ingestion of the neonicotinoid thiacloprid has also been shown to alter gut microbiota composition and disrupt intestinal barrier function, resulting in an increase of harmful substances in the liver (Han et al., 2023). Overall, these alterations may result in reduced individual nutrient absorption and greater susceptibility to diseases with ultimate consequences on survival.

f. Alteration of the nervous system and behavioural consequences

There is increasing evidence of the adverse effects of neonicotinoid insecticides on the nervous system of farmland birds, although this class of pesticides was assumed to have lower effects in vertebrates due to the reduced prevalence of the neural receptor they target (Gibbons et al., 2015). Reduced or enhanced neural functions are reported in Japanese quails and rock pigeons *Columba livia domestica* after ingestion of imidacloprid (Abu Zeid et al., 2019; Rawi et al., 2019) which can result in changes in activity and behaviours (Berny et al., 1999). Ingestion of imidacloprid in white-crowned sparrows *Zonotrichia leucophrys* induced a reduction of feeding and delayed departure from migratory stopover (Eng et al., 2019), and altered their orientation capability (Eng et al., 2017). Nonetheless, other pesticides than neonicotinoids may also interfere with neurotransmitters and result in increased or decreased aggressiveness, territorial and nest defence, and altered parental care and incubation behaviours (Moreau et al., 2022a and references therein). Grey partridges exposed to pesticide mixtures from conventional grains had higher behavioural activity while conversely, passerine birds captured in 'conventional' hedgerows exposed to pesticides had reduced vitality compared to birds fed with organic grains or captured in organic hedgerows respectively (Moreau et al., 2021; 2022b). Altogether, these behavioural alterations may impede birds' migration or survival to migration or predation, which may further affect the population's dynamic (**Figure 6**).

g. Reproductive, parental and transgenerational effects

Reductions in colouration of secondary sexual traits were reported in red-legged partridges, although it depended on the pesticide, the dose and the time of exposure (Lopez-Antia et al., 2013; 2015a; 2015b; 2018; 2021), and in grey partridges exposed to pesticide mixtures (Moreau et al., 2021). The endocrine disruption associated with the HPG axis resulting in altered secretion of steroid hormones may affect the production of sexual ornaments as carotenoid-based colourations (Moreau et al., 2021; **Figure 6**). In the red-legged partridge, lower levels of steroid hormones were found in males exposed to fipronil (for testosterone only, Lopez-Antia et al., 2015b) and in both sexes exposed to tebuconazole (for oestradiol only, Fernández-Vizcaíno et al., 2020) compared to control birds. This may, in turn, alter not only ornamental colouration, with consequences on birds' mate choice and reproductive investment, but also spermatogenesis, and reproductive behaviours. For instance, the sperm density of house sparrows is reduced after exposure to the neonicotinoid acetamiprid (Humann-Guilleminot et al., 2019; **Figure 6**). Neonicotinoid exposure also alters the ovaries of Japanese crested ibis *Nipponia nippon* (Hoshi et al., 2014). Delayed egg laying is observed in red-legged partridges fed with seeds treated with imidacloprid (Lopez-Antia et al., 2015a), thiram Lopez-Antia et al., 2015c) or flutriafol (Fernández-Vizcaíno et al., 2020). In this species, thinner eggshell, reduced size of eggs and fertility with lower hatching rates and higher mortality of offspring are also observed, although the effects vary according to the substance and dose applied (Lopez-Antia et al., 2013; 2015c; 2018; 2021). The reduced breeding success resulting from these pesticide sublethal effects may further participate in the population's decline (**Figure 5**).

Besides these direct consequences on the reproductive output of birds, chicks' morphology, physiology and behaviour may be influenced by parental effects, notably through the maternal transfer of hormones, nutrients and pesticides to the egg (**Figure 6)**. It has notably been found that parents' exposure to pesticides may alter eggs' components (cholesterol, vitamins) (Fernández-Vizcaíno et al., 2020; 2024) and may result in developmental alterations in embryos and hatchlings (Kitulagodage et al., 2011; Ruuskanen et al., 2020c). In red-legged partridge, parental consumption of seeds treated with either imidacloprid, fipronil or thiram results in depressed immune responses in chicks (Lopez-Antia et al., 2015a; 2015b; 2015c). Growth patterns of unexposed offspring can also be altered due to parental effects (Lopez-Antia et al., 2015c; Gaffard et al., 2022a). Recent findings on changes in gene expression of individuals exposed to triazole fungicides and of their first- and second-generation offspring, provide evidence for the possible transgenerational effects of pesticides that could affect populations' viability beyond the exposure timeframe (Fernández-Vizcaíno et al., 2024). Overall, these alterations to nestlings' health may impede their fledging success and survival, especially if they have to migrate and thus need to be in great condition to survive.

h. Complex effects

It appears that for a given species, contrasted sex- and age-dependent sublethal effects may arise depending on the pesticide (or mixture) to which they are exposed. Besides, trade-offs between different functions of the organisms are clearly occurring so that predictions on the effects on one trait are complicated to raise. Altogether, the sublethal effects of pesticides may alter multiple morphological, physiological and behavioural traits of wild birds, with consequences on their survival, reproduction, emigration, and on their offspring through parental effects and eventually transgenerational effects. These effects might ultimately affect population trends (**Figures 5 and 6**). However, there are still knowledge gaps from *in natura* studies to understand how pesticide sublethal effects may be linked to farmland birds' decline.

4. Knowledge gaps from pesticide contamination and sublethal effects on farmland birds *in natura*

From the various aforementioned studies, three main knowledge gaps can be raised: (a) cocktail effects are not considered, (b) mainly domestic and game birds are represented, and (c) experimental conditions do not reflect what happens in the wild.

a. The need to consider cocktail effects

There are various knowledge gaps concerning the fate and effects of pesticides on wild species, and notably farmland birds. Indeed, little is reported about the presence of pesticide mixtures within wild birds and how these mixtures vary across time (but see Peris et al., 2023) and during their lives. The transport, metabolisation, and excretion of pesticides within organisms are studied using the toxicokinetics of chemicals. However, the first limitation of these studies is the investigation of the fate of one substance at a time, generally after its ingestion (Bean et al., 2019; Pan et al., 2022), neglecting both the potential interaction between molecules and the inhalation/dermal absorption kinetics (Smith et al., 2007). This could lead to over or underestimating the pesticide metabolisation and excretion time.

The same applies to studies investigating the sublethal effects of pesticides that usually investigate one (class of) pesticide at a time (Moreau et al., 2022a). Yet, wild birds are exposed to multiple pesticides present in the environment that might together have different effects than the ones observed individually. Pesticides in a mixture can have additive toxicity (generally the case when they have similar mechanisms of action) or interactive toxicity that might be either greater (synergism) or lower (antagonism) than the additive toxicity (Hernández et al., 2017). Therefore, experimental studies investigating the effects of single molecules do not consider the combined effects of a mixture. It appears that evidence for the so-called cocktail effects of pesticides under field conditions is lacking (Moreau et al., 2022a). Moreover, there is a bias in the effects investigated depending on the type of pesticides. Indeed, most studies on the sublethal effects of pesticides concern insecticides and fungicides while the effects of herbicides on birds mainly concern indirect effects rather than direct sublethal effects (Fritsch et al., 2024).

b. Beyond standard laboratory species

Toxicokinetic studies are principally done on domestic species and there is a lack of data concerning wild species (Kuo et al., 2022). It is thus unclear to what extent the toxicokinetic characterization of pesticides is relevant to pesticides' fate within wild birds. Besides the restriction of OECD standard tests for the risk assessment procedure in the EFSA regulation to use standard species for toxicity tests (preferably Japanese quail or Bobwhite quail, EFSA, 2023), experimental studies describing sublethal effects generally consider model species of only a few orders of birds such as Galliformes (partridges, quails) or Passeriformes (sparrows, finches)

(Gibbons et al., 2015; Moreau et al., 2022a). Still, field studies describing pesticide contamination and adverse effects generally include a wider range of Charadriiformes (gulls, terns), Pelecaniformes (herons), and Accipitrifomes (hawks, kites) species for instance (Moreau et al., 2022a). Concerning raptors, one of the first taxa concerned by the sublethal effects of legacy organochlorine insecticides, there has been no study describing individual effects induced by currently used pesticides since 2000 (Fritsch et al., 2024).

c. *The flaws of experimental conditions*

It is often claimed that laboratory studies lack realism, notably because the dietary, social and housing conditions of captivity may influence the responses observed and thus limit extrapolations to wild species (but see Moreau et al., 2022a for examples of field studies). For instance, the environmental conditions experienced in the wild would induce a natural variation of the microbiome that would otherwise not be found in captive birds (Hird, 2017) with repercussions on the possible adaptation to polluted environments (*ARTICLES 7 and 8 in Appendices*). Besides, both toxicokinetics and toxicity tests focus on a short time frame (from a few hours to a month) so that the fate of pesticides and their consequences from long-term exposure are unknown.

d. *How to fill these gaps in field studies?*

Although the EFSA recommends including field studies in the third-tier risk assessment of pesticides with some requirements and recommendations (such as the justification of the area and period studied, and a description of landscape characteristics, agricultural practices, and meteorological conditions), it does not provide clear instructions on how to perform them. Besides, there have been several limitations to the implementation of field studies to assess the fate and effects of pesticides on wild farmland birds. First, technical means to screen simultaneously multiple pesticides of different classes were missing (Millot et al., 2015) or at least available only for some matrices that would need dead or sacrificed individuals (collected eggs and organs for instance; Taylor et al., 2013; Millot et al., 2015; Bro et al., 2016; Rial-Berriel et al., 2021). This type of analysis is also generally expensive so that investigations need important financial support. Moreover, under natural conditions, the studied species face multiple stressors that need to be considered when assessing the effects of pesticides. Different ethical considerations need thus to be addressed when implementing experimentations, especially in the case of protected species such as raptors (Palmer & Greenhough, 2021). Nonetheless, now that non-destructive multi-residue analyses are available (Rial-Berriel et al., 2020; Rodrigues et al., 2023) and that non-invasive methods are developing (Fernández-Vizcaíno et al., 2023), field studies should consider the assessment of wild birds' health in relation with their pesticide contamination. This would be particularly relevant for farmland birds benefiting from long-term monitoring in areas with detailed knowledge of the landscape, soil occupancy and agricultural practices.

The present doctoral work aims to fill these knowledge gaps by assessing the contamination with multiple pesticides (up to 116 substances) and cocktail effects of pesticides on farmland birds monitored under real wild conditions in a study area dominated by intensive agriculture.

IV. Thesis aims and organization

An important issue of my thesis work was to assess the potential role of organic farming as a conservation tool for farmland birds using an integrative approach to multiple organism's functions. Indeed, research on this production system has mainly focused on its effects on biological diversity or farmland birds' reproductive output. Nonetheless, this mode of production excludes the use of synthetic inputs and might thus mitigate their potential adverse effects, and it also encompasses different practices that could be both beneficial (more resources notably) and detrimental (more disturbances) for wild farmland birds. Thus, my work aimed first to assess the effects of organic farming on the global health status of farmland birds. Then, the second step was to assess the actual contamination with pesticides of nestlings reared in an intensively managed agricultural land. The aim was to provide *in natura* evidence of the prevalence, factors of influence, and sublethal effects of pesticide cocktails on farmland birds, screening up to 116 substances in blood. More precisely, my research focused on Montagu's harrier nestlings monitored in the Long-Term Socio-Ecological Research Network, the Zone Atelier Plaine & Val de Sèvre (LTSER ZAPVS). Montagu's harrier chicks are particularly relevant to study pesticide cocktails, as they are "naïve" individuals, hatched on the ground of crops and exposed all along their rearing period to pesticides present in their environment. Besides, being at the top of the trophic chain, the species might experience biomagnification processes and is thus a relevant bio-indicator of the ecosystems' health. A detailed description of the species and the general methods used for my thesis are provided in the following section "General Methods".

In **CHAPTER I**, taking advantage of a large dataset of 380 Montagu's harrier chicks monitored between 2016 and 2021 and of a local database of georeferenced organic crops, I used 12 different life-history traits to assess the potential effects of the proportion of organic farming surrounding nests on chicks' health (*ARTICLE 1*).

CHAPTER II of this work aims to provide (i) a description of pesticide contamination patterns in 55 Montagu's harrier chicks blood-sampled in 2021 (*ARTICLE 2*); (ii) an investigation of the intra and inter-brood variability in contamination levels of these chicks, and of the individual (sex, hatching rank) and spatial (farming system in the surroundings) factors affecting their contamination levels (*ARTICLE 3*); and (iii) of the influence of temporal (age, sampling date) and body condition factors on the pesticide contamination levels of 35 chicks blood-sampled twice during their growth in 2018 (*ARTICLE 4*).

CHAPTER III aimed to contribute to the front of science concerning the sublethal effects of pesticide cocktails on wild farmland birds, through the study of 35 Montagu's harrier nestlings monitored in 2018. I focused on the nestling's physiological stress (measured through corticosterone concentrations) and growth (skeletal, mass and body condition) to confront these traits with their pesticide contamination levels determined two times during their growing period (*ARTICLE 5*).

Lastly, I provide a general discussion of the results obtained in the studies presented in the three chapters. Both the limits of my work and its prospects are given in that section.

GENERAL METHODS

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My three-year research was conducted within the long-term monitoring program of the Montagu's harrier population of the Zone Atelier Plaine & Val de Sèvre (ZAPVS). Therefore, all the studies included in this manuscript rely on a common basis described hereafter.

I. The Zone Atelier Plaine & Val de Sèvre (ZAPVS)

1. History and description of the ZAPVS

The ZAPVS, located in south-western France, in the department of Deux-Sèvres, originated in 1994 with the delimitation of an area of ~12 km² at the instigation of Vincent Bretagnolle (Bretagnolle & Tardieu, 2021). The first aim of this area was to monitor and protect a bird of prey, the Montagu's harrier. This zone increased the following year to attain 250 km² (**Figure 7**). The soil occupancy of the ZAPVS has been determined every year since 1996; first, fields were hand-drawn before the Geographical Information System (GIS) database of the zone was built in 1999 (Bretagnolle & Tardieu, 2021). Agricultural fields of wheat, oilseed rape, sunflower and corn predominantly covered the zone. Farms were, on average, 50 hectares and meadows and extensive livestock farming were still present. Within a decade, the surface of meadows disappeared, and farms are now ca. 120 hectares. Since 2003, more than half of the area has been classified Natura 2000 site, benefiting from the Special Protection Area designation that aims to protect habitats and species representative of European biodiversity. This led to the special protection of 17 bird species, including the little bustard and the implementation of agri-environmental measures. These measures include the absence of alfalfa mowing in spring, the reduction of synthetic inputs, or the establishment of meadows instead of cereal crops, for which farmers get financial support. The ZAPVS attained its actual size of 450 km² (435 km² without the forest of Chizé) in 2005 (**Figure 7**) and was officially labelled "Zone Atelier" in 2008 (Bretagnolle & Tardieu, 2021). The 15 Zones Ateliers included in the French network are Long-Term Socio-Ecological research (LTSER) platforms that aim to study the interaction between human society and its ecosystem (i.e., the socio-ecosystem) locally. Nowadays, the ZAPVS is dominated by intensive agriculture, with the main crops remaining wheat (average of ~34% of the area between 2009-2016), corn (~10%), sunflower (~10%) and oilseed rape (~8%) (Bretagnolle et al., 2018b). Meadows (including permanent grasslands and temporary hay) decreased drastically to cover 15% in 2016. Less than 10% of the ZAPVS is urbanised, and ~3% is covered by deciduous forest fragments (Bretagnolle et al., 2018b). Crops grown organically are also georeferenced during the yearly determination of soil occupancy, providing a precise and unique knowledge of the land uses in the ZAPVS. Organic farming covered ~18% of the surface in 2021.

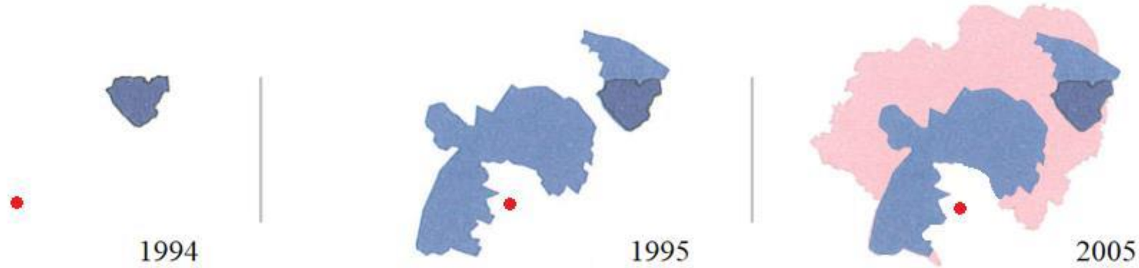

Figure 7. Changes in the size of the Zone Atelier Plaine & Val de Sèvre (ZAPVS) between 1994 (12 km²) and 2005 (450 km²). The red dot corresponds to the localisation of the Centre d'Études Biologiques de Chizé. Modified by T. Schneider-Bruchon, from Bretagnolle & Tardieu (2021).

2. Biodiversity monitoring

Since the creation of the ZAPVS, biodiversity surveys have been carried out every year. First, bird and small mammal surveys started in 1994, then arthropods the following year, plants since 2005 and soil organisms since 2016 (Bretagnolle et al., 2018b). Among the taxa included in recent studies realised in the ZAPVS, plants (Henckel et al., 2015; Berquer et al., 2021), earthworms (Pelosi et al., 2021), pollinators (Requier et al., 2015; Gay et al., 2024), carabid beetles (Marrec et al., 2015), small mammals (Lambin et al., 2006; Fritsch et al., 2022), passerine birds (Brodier et al., 2014; Moreau et al., 2022b) and bird of prey (Rabdeau et al., 2021) are found. Sampling designs are adapted from year to year depending on the taxon and the research objectives. Bird monitoring, for instance, started with the exhaustive search of harrier and little bustard nests and point counts for passerine birds. The monitoring of these species is still ongoing, but the protocol of point counts has varied since 1996, adding point counts or increasing the counting time for instance. This long-term monitoring permits precise estimations of the birds' species and abundance present in the area every year and, therefore, to estimate population trends. These unique long-term trends enable evidence of the decline of farmland birds in the area within 26 years (Schneider-Bruchon et al., 2023).

II. The Montagu's harrier, *Circus pygargus*

1. Distribution and trends

The Montagu's harrier is a migratory small bird of prey (**Figure 8**), distributed across Europe during the breeding season and wintering either in western Africa (western European populations) or India (eastern European populations) (García & Arroyo, 1998; Limiñana et al., 2012). Individuals travel ~252-296 km per day for 8 to 30 days during their migration trip (Limiñana et al., 2012; Schlaich et al., 2017). Generally, individuals leave their wintering site in mid-March and arrive at their breeding site in April, where they breed in semi-colony from May to August. Juveniles and adults gather in roosts by the end of August and leave their breeding grounds through migratory waves until mid-September (Limiñana et al., 2012). Outside the breeding season, Montagu's harriers gather in communal roosts at night with up to hundreds of birds (Arroyo et al., 2004). A mixed residency-itinerant strategy is observed during their wintering period, with a few birds having a single wintering site and most using approximately three different sites during winter (Schlaich et al., 2023).

The species is not considered as threatened at the global level, but the declining trends of some of its populations lead to consider it as vulnerable or in danger locally. In France, populations have declined by 10–30% over the past 30 years (Millon & Bretagnolle, 2004; Comolet-Tirman et al., 2015), with agricultural intensification considered as the main responsible for this trend (Butet & Leroux, 2001; Arroyo et al., 2002; Arroyo et al., 2004; Santangeli et al., 2015). Indeed, although the mortality rates in Montagu's harriers are higher during migrations than during breeding or wintering, probably due to unfavourable conditions at stopovers, the main reason for the declining trends is probably the low recruitment rates due to low breeding success in some years (Arroyo & Bretagnolle, 2000; Arroyo et al., 2002; Schlaich et al., 2017). This low breeding success has been attributed to predation and reduction in food abundance, but more importantly to agricultural practices (Butet & Leroux, 2001). As nests are settled on the ground, they can be destroyed during mowing and harvesting. A 10-year monitoring in France revealed that human protection of nests enabled 58% of chicks to fledge. Therefore, conservation measures are essential to maintain sustainable populations (Bourrioux et al., 2017; Arroyo et al., 2002). Another potential threat linked to agricultural practices would be the use of synthetic pesticides, as it can reduce the food availability (notably grasshoppers in wintering sites; Bourrioux et al., 2017) and contaminate both the direct environment and the prey consumed by the species, leading eventually to sublethal effects.

Figure 8. A Montagu's harrier flying. Photo credit © S. Phelippeau

2. Diet composition

The Montagu's harrier is a specialist raptor, feeding on the most productive prey locally (Arroyo et al., 2004). It preys upon voles, mainly *Microtus arvalis,* for the population monitored in the ZAPVS and can also prey upon orthopteran insects, notably grasshoppers and crickets, in years of poor vole abundance (Thiollay, 1968; Salamolard et al., 2000). Other alternative prey includes small birds, especially passerines, lagomorphs, notably juvenile hares, and exceptionally other classes of insects, reptiles or eggs of ground-nesting birds (Salamolard et al., 2000; Arroyo et al., 2004). The relative proportion of each prey varies depending on the region and the season (Arroyo et al., 2004). In north-central Kazakhstan, lizards represent more than half of the prey (54%) found in pellets and observations, then small mammals (17%), birds (14%) and insects (14%), although in terms of biomass, birds represent the most important prey (39%) (Terraube et al., 2010). In central Spain, the diet of the species is more generalist, with either insects (up to 58%) or lagomorphs (up to 61%) being the most important prey, followed by birds (up to 32%) and small mammals (up to 23%) (Arroyo, 1997; García & Arroyo, 2005). These proportions vary along the breeding season, with bird and insect proportions increasing by the end of the rearing period (García & Arroyo, 2005). In south-western France, Montagu's harrier diet is composed of 34-87% of voles (in biomass), depending on their abundance and the period of the breeding season, increasing from the pre-laying to the fledging period (Salamolard et al., 2000). In that region, the second highest biomass in Montagu's harriers' diet is insects (crickets 1-24% and Odonatae 0.2-27%), while birds constitute 2-15% of the total biomass (Salamolard et al., 2000). Montagu's harriers' diet in winter is poorly documented, but in western African sites, it includes mostly grasshoppers and ground birds (Arroyo et al., 2004).

3. Reproduction

The Montagu's harrier is a monogamous species, with pair bonds lasting for a breeding season, although polygamy may occur (Arroyo et al., 2004). Aerial displays, which consist of an ascendant flight and a rapid headlong dive towards the ground accompanied by undulations, loops, twists and calls, are performed by both sexes throughout the breeding period (Arroyo et al., 2013). Sky-dancing displays would be used for reciprocal mate choice (both male and female choice) (Arroyo et al., 2013), and cere colouration would also be a sexual signal, with carotenoid-based pigmentation being indicative of a mate's quality (Mougeot & Arroyo, 2006).

The Montagu's harrier is a sexually dimorphic species, with females being bigger (~15% heavier; Leroux & Bretagnolle, 1996) than males, and with different plumage, females being brown and males grey when sexually mature (**Figure 9**). The sexual dimorphism becomes apparent in chicks during the second half of the rearing period (Millon & Bretagnolle, 2005), although the plumage of juveniles stays brown until attaining sexual maturity (2-3 years, still, successful breeding for one-year males may occur; Arroyo, 1996). Nestlings can be sexed once they are ~15 days old thanks to the colour of their iris (brown for females and grey for males; Leroux & Bretagnolle, 1996).

Figure 9. Sexual dimorphism in Montagu's harriers: female (left) and male (right). The male was wing-tagged with coloured marks (visible on its back) by Etienne Debenest, the referent in the Deux-Sèvres department of the national harrier program, licensed by the Centre de Recherches sur la Biologie des Populations d'Oiseaux (CRBPO; Museum National d'Histoire Naturelle). Photo credit © S. Phelippeau

The species is a ground-nesting bird of prey, building small nests (Ø ~20 cm; **Figure 10**) using a few nest materials. The species breeds in solitary or in semi-colony in which the number of neighbouring nests can attain 21 within 600 m with a distance between nests as low as 15m, but semi-colonies of four to six nests distant of ~155-220 m are more common (Arroyo et al., 2004). Nest defence efficacy is greater for semi-colonies than isolated pairs due to a greater number of mobbers harassing predators (Arroyo et al., 2001).

Figure 10. A nest of Montagu's harrier with four eggs settled on the ground of a wheat field. Photo credit © E. Fuentes

In Montagu's harrier, a bi-parental care of nestlings is ensured. Males are responsible for food-provisioning to both incubating females and nestlings, while females defend nests, protect chicks from adverse weather conditions and ensure thermoregulation (Kitowski, 2003; García & Arroyo, 2005). Females increase their participation in food-provisioning by the end of the rearing period, although they can adjust their participation depending on food availability (García & Arroyo, 2005). Besides, males hunt several kilometres away from the nest, while females usually hunt smaller prey near the nest (García & Arroyo, 2005). The incubation period lasts 29 days, and nestlings are reared until they are 30-35 days (Arroyo et al., 2004; **Figure 11**). The breeding success of the species relies on the availability of its main prey (besides the influence of nest destruction from agricultural activities and predation). In Europe, the mean productivity (number of fledglings per breeding attempt) varies between 1.1 and 2.7 (Arroyo et al., 2004). In south-western France, depending on the availability of common voles, the mean productivity is 2.05 fledglings per year, although pairs can raise up to five nestlings per breeding season.

Figure 11. A nestling of Montagu's harrier aged ~26 days old, on its nest, on the ground of a wheat field. Photo credit © S. Phelippeau

III. Monitoring and experimental design

In the following sections, I describe how the monitoring of Montagu's harrier nests and the handling of nestlings is generally performed every year. My contribution to each of these steps is stated in the "Preface" section of the manuscript.

1. Nests' monitoring

At the start of the breeding season, reproductive indices (aerial displays, prey passes, coupling) were searched around the ZAPVS to identify potential semi-colonies and lone couples. Nests were located first using binoculars by sighting females landing on crops with nest material or prey delivered by the male (**Figure 12**). Nest location was then confirmed either by walking in the crop or by droning the suspected location, and registered using Global Positioning System (GPS) coordinates.

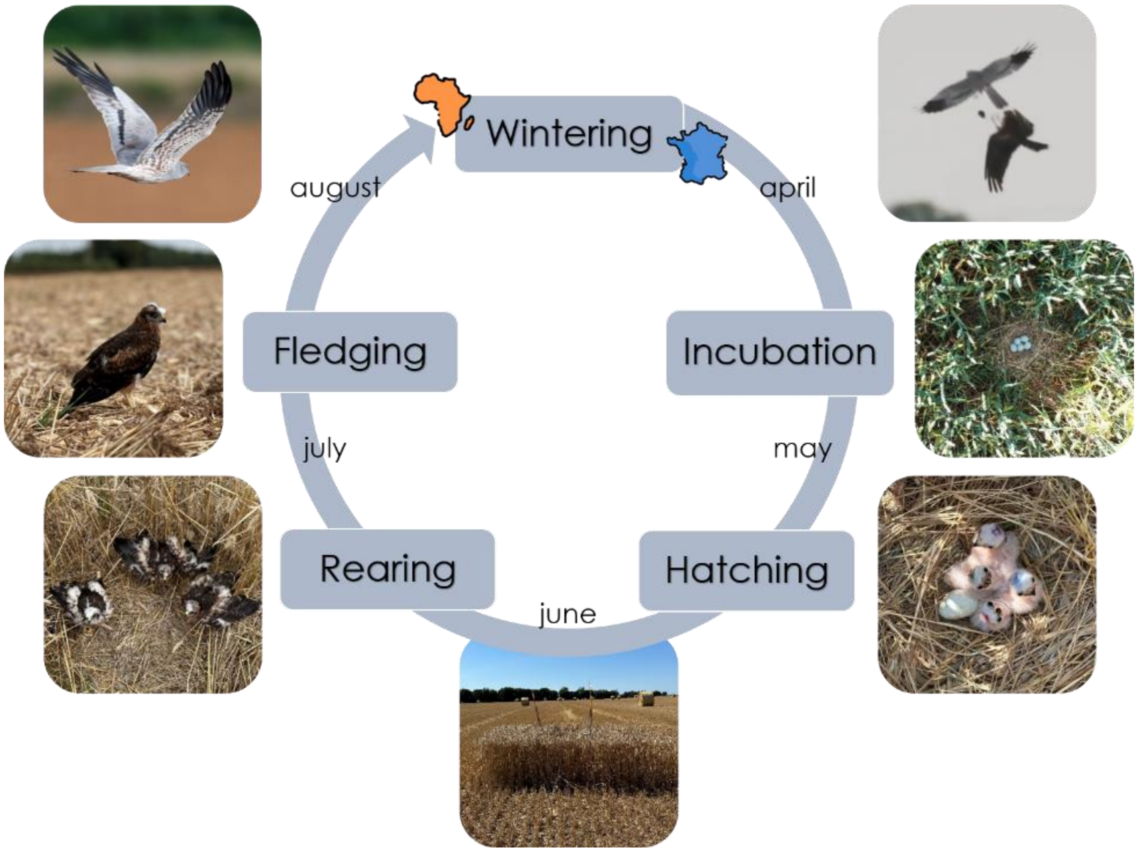

Figure 12. Monitoring of Montagu's harrier reproduction in the ZAPVS.
Photo credit © E. Fuentes, except top left photograph © C. Ingrand.

Nests were visited once or twice before hatching to record the final clutch size. The hatching date was estimated from the eggs' density (Arroyo et al., 2017) by measuring the eggs' length and width using a calliper (accuracy ± 0.1 mm) and by weighing them with a Pesola 500 g digital scale (accuracy ± 0.1 g). Nests were visited every week after hatching until chicks fledged, thus depending on the brood size and the age gap between chicks, 4 to 5 visits per nest were carried out (lag time between visits of 7 ± 2 days; **Figure 12**). During these visits, chicks were at least weighed and other morphometric, physiological and behavioural measures were also performed depending on the age of the chicks and the year (see section "*III.2. Field* *data collection*"). Individual identification of nestlings was first realised by head-marking chicks with a water-based colour pen until they were ~15 days old when they were banded with an aluminium ring provided by the Museum National d'Histoire Naturelle (MNHN) de Paris (France) engraved with a unique code.

To protect Montagu's harrier nests, a wire netting around each nest was installed during one of the visits, depending on the harvest date (**Figure 12**). This delimitation of ~1m^2 allows to prevent chicks from dispersing around the nest and being crushed during crop harvesting. This also prevents terrestrial predation. This protection was taken off when checking that the chicks had fled successfully.

2. Field data collection

The general timeline followed during the chicks' handling is represented in **Figure 13**. This corresponds to the main steps included in the experimental design that was set up in 2016 for nestlings aged ~26 days old (± 2 days; **Figure 11**), although some yearly adjustments to the protocol have been made depending on the study objectives (see details in the section "*IV. Summary of the data analysed*").

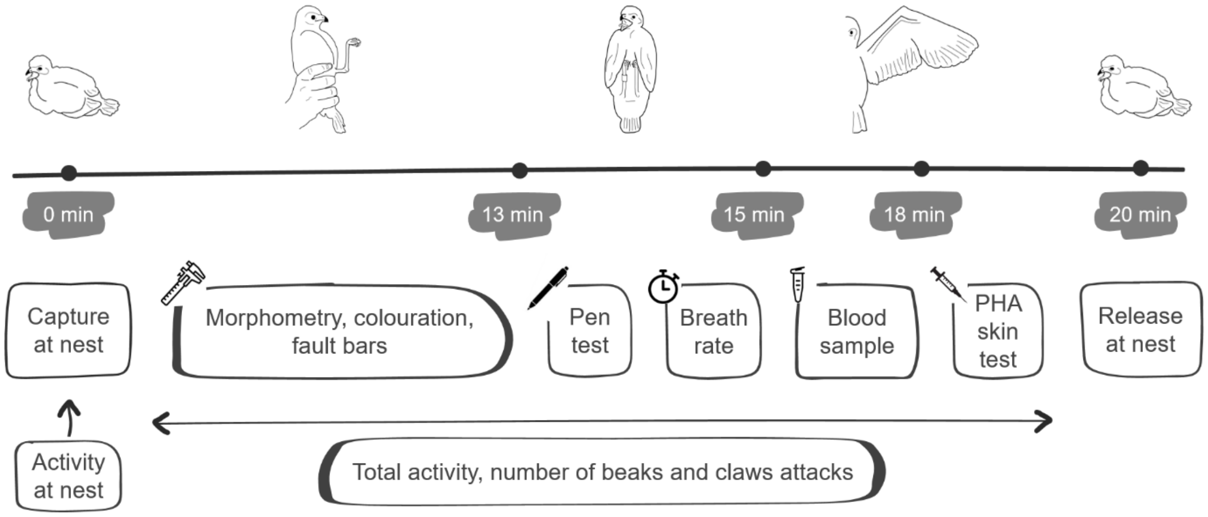

Figure 13. Experimental design during Montagu's harrier handling. Details on each life-history trait measured are provided in the corresponding sections.

a. *Behavioural tests*

The behaviour of an organism results from a range of biochemical and physiological processes and is considered to reflect an individual's health (Clotfelter et al., 2004). Behaviour is often considered a more sensitive response to stress or toxicity levels than other life-history traits (Grue et al., 1997; Clotflelter et al., 2004; Hellou, 2011; Moreau et al., 2022a). Indeed, behaviour may present subtle alterations in response to low toxicity levels, being for instance 10-1000 times more sensitive than the LD50 value (Hellou, 2011). Following the work of J. Rabdeau (Rabdeau et al., 2019), the behaviour of nestlings was assessed both when the experimenter arrived at the nest and during the handling of chicks. A score between 0 and 2 was attributed according to the activity of the nestling at the nest: when it was standing and did not move = 0; when retreating = 1; and when running away = 2. Once caught at the nest, the behaviour of chicks was assessed throughout handling and scored between 0 and 3 according to their activity. When the chick was motionless = 0; when moved rarely = 1; when moved regularly = 2; and when moved continuously = 3. The number of beak and claw attacks was also recorded. A pen test was also performed during handling and consisted of passing a pen (unknown object) in front of the chicks' eyes to record if the chick was visually following the object or if it tried to attack the pen (**Figure 14**).

Figure 14. Pen-test realised during chick's handling. Photo credit © S. Phelippeau

b. Morphology

Morphological measures enable the estimation of bird's body condition. Multiple indexes exist to assess the body condition, which is a widely used proxy for bird's health status, reflecting reserves (fat and protein) and other body components in wild organisms (Peig & Green, 2009; 2010). It is thus involved in post-fledging survival, as too high body condition can indicate obesity (wing overloading), and too low body condition a lack of energy that can both impede chicks to fledge properly (Wright et al., 2006).

Nestlings were weighed with a Pesola 500 g digital scale (accuracy ± 0.1 g) during the first post-hatching visit, and with a spring scale (accuracy ± 5 g) during further visits. The chicks' tarsus length was measured with a digital calliper (accuracy ± 0.1 mm) and wing length with a ruler (accuracy ± 1 mm) (**Figure 15**). Generally, the right limbs of chicks were measured once or twice during the first weeks post-hatching and the left limbs were measured twice only for the last visit. At the last visit, tail length was also measured with a ruler.

Figure 15. Measure of tarsus length with a calliper (top) and of wing length using a ruler (bottom), of a Montagu's harrier nestling. The aluminium ring provided by the MNHN allowing individual identification can been seen on the tarsus of the nestling. Photo credit © S. Phelippeau

c. *Colouration*

The carotenoid-based colouration of bare parts of raptors is a secondary sexual trait that provides an indication on the quality of the individual (more intense colouration signalling an individual of good quality; Bortolotti et al., 2000; Mougeot & Arroyo, 2006). In Montagu's harrier nestlings, these ornaments are likely to play a role in communication with parents and among siblings (Sternalski et al., 2011; 2012). Colouration may be limited by the availability of circulating carotenoids as these are not synthesized *de novo* but are provided by the diet (Sternalski et al., 2010). Trade-off processes with other physiological functions (immunity, oxidative stress) that require carotenoids might also influence the colouration (Sternalski et al., 2011; García-Heras et al., 2017).

The colouration of the bare parts of Montagu's harrier nestlings (cere and tarsus) was assessed using a colourimetric chart from Yolk Colour Fan (Roche, Neuilly-sur-Seine, France). Scores ranging from 0 (pale yellow) to 15 (orange) were attributed to the bare parts through direct visual comparison with the chart (**Figure 16**), as these scores are highly correlated with colouration estimated from a spectroradiometer (Faivre et al., 2001).

Figure 16. Scoring the colouration of the tarsus of a Montagu's harrier nestling using a colourimetric chart. Photo credit © S. Phelippeau

d. Fault bars

Fault bars are bands of abnormal or missing barbules on the part of the feather or the entire feather that arise from stressful conditions during feather formation (Jovani & Rohwer, 2016). A large variety of stressors might induce fault bars, including shocks from unsuccessful predation events, chronic psychological stress (such as increased predation risk due to reduced vegetation cover) or physiological stress (due to infections, anthropic disturbances or pollutants, for instance) (Jovani & Rohwer, 2016; Ganz et al., 2018; Rabdeau et al., 2023). A fault bar is visually detectable as a translucent zone of the feather that is likely to break, that can be light, moderate or severe (fine to wide bands; **Figure 17**). Broken feathers cannot be replaced until the next moult, fault bars can thus reduce flight performances with ultimate consequences for survival (Jovani & Rohwer, 2016). The number of all fault bars detected on the most affected rectrix feather of the chick's tail was counted and reported (**Figure 17**).

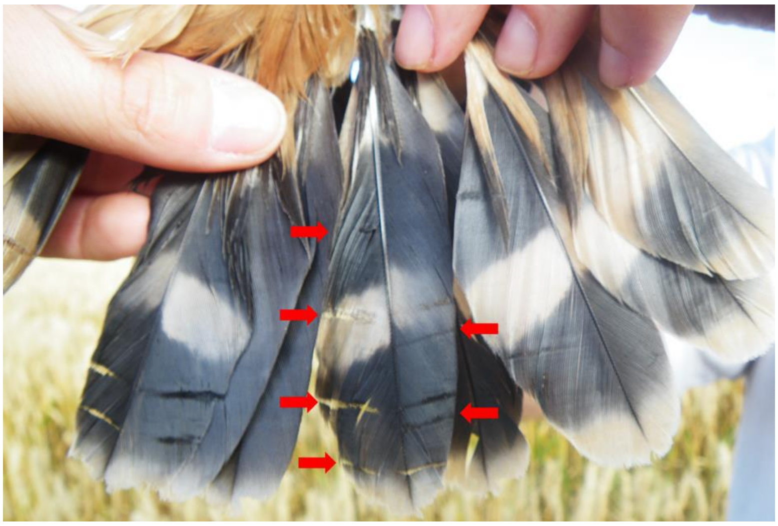

Figure 17. Fault bars on rectrices of Montagu's harrier nestlings. Red arrows indicate fault bars on the most affected rectrix. Photo credit © K. Monceau

e. Breath rate

The frequency of respiratory acts responds to stressful conditions just as the cardiovascular system and has the advantage of being easier to obtain in wildlife (Carere & Van Oers, 2004). Thus, the physiological stress of birds can be assessed through their breath rate (Torné-Noguera et al., 2014; Rabdeau et al., 2019). Chicks were handled in back position and the number of thoracic movements was counted for 15 s three consecutive times (45 s in total). The average of the three counts was reported as the breath rate score of a chick.

f. Blood sampling and subsequent analyses

For each blood sample, a sterile needle was used to puncture the brachial vein of nestlings, and heparinized capillaries were used to collect blood into plastic microcentrifuge tubes (**Figure 18**). The volume collected varied according to the analyses planned (and never exceeded 500 µL). Samples were stored and transported to the laboratory in a cooler (0–5 °C). Then, samples were either centrifuged to extract plasma or directly stored at -20 °C for further analyses (see below).

Figure 18. Blood sampling from the brachial vein using a heparinized capillary. Photo credit © S. Phelippeau

i. Erythrocyte sedimentation rate and haematocrit

Both the sedimentation rate of blood cells and haematocrit are proxies of general health status and humoral immunocompetence in birds (Saino & Møller, 1996; Ots et al., 1998). The sedimentation rate depends on cell and plasma factors and increases due to an increase of fibrinogen and gamma-globulin in case of infectious and inflammatory diseases (Saino & Møller, 1996). The haematocrit (or packed cell volume) reflects the extent of oxygen uptake and transfer to tissues, and can be considered proportional to metabolic activity in the days/weeks preceding the sample (Ots et al., 1998). Low values indicate anaemia and may suggest bacterial infections and gastrointestinal disorders (such as parasitism), or nutritional deficiencies (Ots et al., 1998).

For each nestling a single microcapillary of 10 µL was collected from blood sampling to determine both erythrocyte sedimentation rate (ESR) and haematocrit (**Figure 19**). The microcapillary was left in a vertical position for 5 hours in a cooler (0-5 °C). The height filled by erythrocytes and the total height of whole blood (erythrocytes and plasma) were measured using a digital calliper (accuracy ± 0.01 mm) to calculate ESR which is the proportion of blood cells sedimented per hour. Once ESR was measured, the microcapillary tube was centrifuged at 5000 rpm for 5 min to measure again the height of blood cells and total height using the digital calliper (**Figure 19**). Haematocrit corresponds to the proportion of the tube filled with erythrocytes divided by the total volume of blood sample in the tube, given as a percentage (Saino and Møller, 1996; see also Biard et al., 2015).

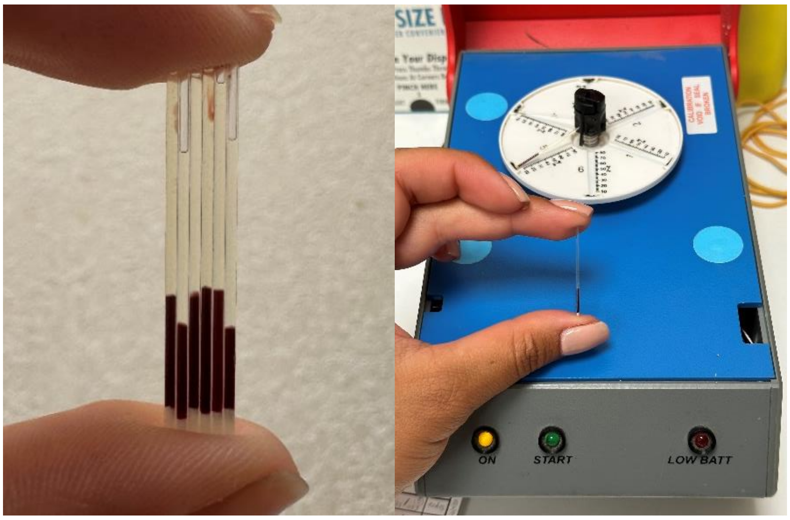

Figure 19. Micro capillaries from six nestlings of Montagu's harriers (left) and centrifugation of the tubes for haematocrit determination (right). Erythrocytes are packed at the bottom of the tube while plasma fills the rest of the tube. Photo credit © E. Fuentes

ii. White blood cells and H/L ratio

Stress and inflammatory processes might increase the number of leucocytes (Ots et al., 1998). Heterophils are leucocytes involved in the inflammatory response which lysis after phagocytosis may be harmful to the tissues (Ots et al., 1998). An increased proportion of heterophils compared to lymphocytes is induced by stress responses, the H/L ratio is thus widely used as an indicator of multiple stressors in avian studies (Ots et al., 1998; Maxwell, 1993; Biard et al., 2015). The H/L ratio is notably considered less variable than individual leucocyte count and more reliable than corticosteroid levels (Maxwell, 1993).

A droplet of nestling's blood was spread on a glass slide with a coverslip to obtain a thin layer of blood (**Figure 20**). Slides were air-dried and transported to the laboratory where they were fixed in absolute methanol and stained with Giemsa solution (Sigma Chemical, St. Louis, MO, USA). A droplet of oil was applied to blood smears to examine them under a microscope (1000x magnification). The number of leucocytes found among 8000 erythrocytes and of the different types of leucocytes (lymphocytes, eosinophils, heterophils, monocytes and basophils) were reported. The proportion of heterophils to lymphocytes (H/L ratio) was estimated from this counting. If present, the number of blood parasites was reported.

Figure 20. Blood smear from Montagu's harrier. Photo credit © S. Phelippeau

iii. Plasma carotenoid concentration

Carotenoids are pigments involved in different functions of birds, including in the colouration of ornamental traits, in detoxification processes as antioxidants, and in the immune system due to their immuno-enhancing properties (Møller et al., 2000; Sternalski et al., 2011). A decreased concentration of circulating carotenoids in blood can result from a humoral and cell-mediated immune response and inflammatory response (Perez-Rodriguez et al., 2008).

A microcentrifuge tube containing ~150 µL of blood was collected for each nestling during the blood sampling and was centrifuged (10 min at 9000 rpm) to collect 20µL of plasma (**Figure 21**). Plasma samples were kept at -20 °C until further laboratory analyses. Plasma carotenoid concentration was subsequently obtained in micrograms per millilitre (µg/mL) using a standardised colourimetric technique (see Biard et al., 2010 for additional information).

Figure 21. Tubes containing red blood cells (left) and plasma (right) after centrifugation of the whole blood sample. Photo credit © E. Fuentes

iv. Acetylcholinesterase activity

Acetylcholinesterase (AChE) is an enzyme involved in regulating the transmission at the nerve synapse. Some neurotoxic pesticide molecules (such as carbamates, and organochlorines) negatively affect AChE activity by altering neural functions, which disrupts diverse behaviours and complex functions (Grue et al., 1997; Eng et al., 2017; Moreau et al., 2022a).

AChE activity in milliunits per minute (mU/min) was obtained using 5 µL of whole blood collected during blood sampling, diluted in 100 µL of a 1% Triton X-100 solution and measured over 40 min at 324 nm and 37 °C (method derived from Ellman et al., 1961). AChE activity was calculated as the maximum change in absorbance per minute during kinetics analysis.

v. Pesticide multiresidue analyses

Pesticide analyses were done at the ICPEES laboratory in collaboration with M. Millet. Briefly, following the methodology described in Rodrigues et al. (2023), 50 µL of whole blood samples (i.e., red blood cells and plasma) were used for pesticide titration by gas chromatography coupled to tandem mass spectrometry (GC/MSMS) and liquid chromatography coupled to tandem mass spectrometry (LC/MSMS) and using multiple reaction monitoring (MRM) for quantification. At present, 116 molecules including 43 herbicides, 38 fungicides, 33 insecticides, a synergist (increasing the actions of pesticides) and a safener (improving herbicide selectivity towards weeds rather than crop plants) among the most used in France are titrated by this method. The main properties, the method of analysis, the limit of detection (LOD) and the limit of quantification (LOQ) are provided in **TABLE A1** in Appendices.

g. Immune system test

Phytohemagglutinin (PHA) is a plant protein, found notably in beans, used as a mitogen to induce the proliferative response potential of circulating T lymphocytes (Smits et al., 1999). The PHA skin test is widely used on avian species to challenge their immune system as the skin-swelling mirrors the pro-inflammatory potential of individuals.

This test was performed through the subcutaneous injection of 0.5 ± 0.1 mg of PHA diluted into 0.1 mL of phosphate-buffered saline (PBS) in the wing patagium as described by Sternalski et al. (2012). The thickness of the patagium was measured three times before injection using a pressure-sensitive dial thickness gauge (spessimeter accuracy ± 0.01 mm; **Figure 22**) and again 24 h after injection. The response to the PHA skin test corresponds to the change in average thickness (in mm) of the injection site, and thus the magnitude of this change reflects the immune potential of nestlings (Smits et al., 1999; see also Biard et al., 2015).

Figure 22. Measure of the thickness of the wing patagium of a Montagu's harrier nestling using a spessimeter. Photo credit © S. Phelippeau

IV. Summary of the data analysed

The global experimental design described in the sections above varied between its onset in 2016 and the end of my work in 2024. For instance, in 2016 chicks' behaviour and breath rate were not assessed. Besides, another blood sample was collected within the first three minutes post-capture from 2017 to 2019 to assess nestling's basal and stress-induced corticosterone secretion (details in **CHAPTER III**). Microbial samples have been collected since 2022 to assess nestlings' gut microbial community (details in *ARTICLES 7 and 8 in Appendices*). Consequently, not all parameters were available for the entire period that I studied in the present manuscript

(2016-2022). Details on the sample sizes of the data used in the different chapters presented in this manuscript are provided in **Table 1**.

Table 1. Summary of the number of nestlings assessed per year for each of the measured parameters. The total number of monitored chicks until fledging is given in brackets below the corresponding year. Cells in light green correspond to data used in **CHAPTER I**, in light orange used in **CHAPTER II**, in light blue for **CHAPTER III** and in grey for studies included in appendices.

Measured parameters	2016 (40)	2017 (32)	2018 (81)	2019 (84)	2020 (85)	2021 (58)	2022 (41)
Activity at nest	0	32	81	82	85	58	41
Total activity	0	32	81	83	84	58	41
Number of beak and claws attacks	0	32	81	83	85	58	41
Pen test	0	32	81	83	84	58	41
Morphometry	40	31	81	84	85	57	41
Cere/tarsus colouration	40	32	81	84	85	58	41
Fault bars	37	25	81	69	84	53	41
Breath rate score	0	32	81	84	84	58	41
Erythrocyte sedimentation rate	35	30	79	77	84	57	40
Haematocrit	26	31	79	77	83	52	40
Plasma carotenoid concentration	37	27	70	52	66	55	38
AChE activity	0	0	74	69	79	49	38
Pesticide titration	0	0	35 (x2)	0	0	5 5	41
Corticosterone concentration	0	31	81	79	0	0	0
White blood cell count	22	30	62	81	80	51	40
H/L ratio	21	30	61	81	80	51	40
PHA skin test	39	30	81	76	74	57	39
Microbial community	0	0	0	0	0	0	22

CHAPTER I

ORGANIC FARMING EFFECTS ON NESTLING HEALTH

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Summary of Chapter I

The intensification of agriculture involved drastic land-use changes and reduced landscape heterogeneity by removing hedgerows and natural/semi-natural elements to obtain large fields of monocultures. It was also characterised by a mechanisation of agricultural practices and the use of synthetic inputs (fertilizers and pesticides) to increase yields. Conventional farming (CF) is often opposed to organic farming (OF), which promotes the establishment of non-cropped habitats and prohibits the use of synthetic inputs, generally increasing labour in OF systems. Various studies compared the species richness and abundance between OF and CF production systems and reported that OF systems promote biodiversity by providing larger amounts of food and shelter to both invertebrate and vertebrate species. Farmland birds whose populations were showing steep declines in both Europe and North America, have been shown to benefit from OF practices, showing higher abundance, diversity and breeding success than in CF fields. However, very few studies considered the potential effects of agricultural systems on farmland bird's life-history traits, and none on their health status, although this represents the underlying mechanism leading to population growth or decline. Indeed, altered life-history traits could impair bird’s survival and reproduction, which in turn affects population dynamics. In this chapter, I aimed to provide an overview of the relative effects of OF compared to CF systems within the same area, on the health of nestlings of a farmland raptor, the Montagu’s harrier. I implemented an innovative approach, considering OF in the habitat surrounding nests as a gradient rather than a factor opposing conventional and organic farms. Because I had no *a priori* on the scale of influence of OF on Montagu’s harrier traits, I estimated the proportion of OF fields around nests at radii ranging from 100 to 2000 m, providing a gradient of potential spatial scales of influence. I investigated the effect of the percentage of OF around nests at the 20 different buffer sizes on multiple life-history traits of nestlings, covering their morphology, physiology and behaviour. More precisely, twelve different traits reflecting the overall health status of nestlings were included: the body condition, colouration of bare parts, fault bars, breath rate, erythrocyte sedimentation rate, haematocrit, carotenoid concentrations in plasma, acetylcholinesterase (AChE) activity, total number of white blood cells, heterophils to lymphocytes (H/L) ratio, phytohemagglutinin (PHA) skin test, and a score of global activity and vigour during handling. In total, 380 Montagu’s harrier chicks from 137 nests monitored between 2016 and 2021 were included in the analyses. The OF crop plots corresponding to the year of chicks’ monitoring were obtained from the Geographical Information System (GIS) database of the Zone Atelier Plaine & Val de Sèvre (ZAPVS). Three traits were related to the proportion of OF around nests: the H/L ratio, the colouration of the cere (carotenoid-based ornament) and the behavioural score of global activity/vigour. While the H/L ratio, which reflects physiological stress in birds, was increasing with the increasing proportion of OF at 600 m around nests, the carotenoid-based ornament, indicative of individual quality, had greater colouration with increasing OF at 1500 m around nests. The increasing H/L ratio indicates that Montagu’s harrier nestlings experience stressful conditions when surrounded by OF fields; eventually due to the greater human disturbances and/or to higher negative interactions with congeners or other raptor species and/or greater exposure to pathogens. Indeed, OF promotes diversified habitats that may be more attractive to recreational human activities and involves more mechanical work that results in repeated human presence, both perceived as a disturbance and increasing chicks' stress. OF may be attractive to other raptor species, increasing the competition for food and even the predation risk of nestlings. This may increase the time parents leave chicks for foraging or protecting the nest. Extended periods of absence of parental care may result in greater stress in nestlings. Besides, contrary to CF systems in

which pesticides are applied and reduce the number/diversity of pathogens, OF systems may increase the exposure of nestlings to pathogens and thus result in higher H/L ratios. Considering that carotenoids are involved in multiple functions of the organism, including detoxification processes and colouration of carotenoid-based traits, and are provided exclusively by the diet, the greater colouration of the cere with increasing OF may be explained by two mechanisms. First, this may indicate that contrary to CF, OF promotes alternative prey, richer in carotenoid content (grasshoppers and passerines), in the diet of Montagu's harriers. An alternative explanation that does not exclude the previous one is the trade-off in the allocation of carotenoids in either the maintenance of the organism or of secondary sexual traits. Exposure to pesticides in CF systems may involve a higher need for carotenoids for detoxification processes, reducing the amount available for the colouration of bare parts or other functions such as the immune system, although we did not find any effect of the farming system on proxies of this trait. Finally, the global activity of chicks was higher when the proportion of OF at 200 m around nests was higher, which may be alternatively explained by the greater exposure of nestlings to pesticides when the proportion of CF is more important and by the greater disturbances experienced by nestlings when the proportion of OF is higher. Neurotoxicity of pesticides may result in the lowered activity of nestlings surrounded by a higher proportion of CF, however, we did not find any effect on the AChE activity, a trait indicative of alterations in the neural functions. On the other hand, greater sensitization of nestlings to humans due to recreational/farming activities may result in greater aggressivity/vigour during handling. Overall, the results of this chapter allowed evidence that the relative effects of farming systems on farmland birds' health are complex and are detectable at different spatial distances. The integration of a wide range of life-history traits and distances enables us to draw complete conclusions. Nonetheless, the role of the natural/semi-natural habitats and pesticide use or other practices have not been disentangled and deserve further investigation. The titration of pesticides in nestlings would notably be a major improvement in assessing the effects of these inputs on farmland birds' health.

ARTICLE 1

EFFECTS OF CONVENTIONAL VS. ORGANIC FARMING PRACTICES ON RAPTOR NESTLING HEALTH: NEITHER BLACK NOR WHITE

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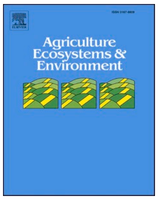

Effects of conventional vs. organic farming practices on raptor nestling health: Neither black nor white

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ABSTRACT

Agricultural intensification is one of the main threats to biodiversity. Farmland bird specialists such as Montagu's harrier, *Circus pygargus,* are particularly at risk and declining. Conventional farming (CF) production systems usually involve landscape homogenisation, mechanisation, and the use of synthetic pesticides that may have direct and indirect effects on individuals. By contrast, organic farming (OF) systems typically promote agro-ecosystem health, which benefits biodiversity and the reproductive success of birds. However, the potential effects of agricultural systems on life history traits of Montagu's harrier chicks have not been investigated. Still, altered life history traits could impair chick survival and future reproductive success, which may in turn impact population dynamics. Here, we investigated the effects of OF (measured as a percentage around nests at different buffer sizes from 100 m to 2000 m) on a set of life history traits covering the behaviour, physiology (haematological, immune and nervous systems) and body condition of 380 chicks from 137 nests monitored between 2016 and 2021. At a local scale (<2000 m), only the H/L ratio (indicative of physiological stress) and carotenoid-based ornaments were clearly related to OF percentage. At 600 m around the nest, a higher OF percentage increased the H/L ratio, suggesting that chicks experienced greater stress due to either increased human disturbance or higher intra-/interspecific negative interactions around OF crop plots. Carotenoid-based ornaments were more strongly coloured with increasing OF around the nest at 1500 m. Considering the role of carotenoids in both detoxification processes and expression of secondary sexual traits, this result may indicate that CF would lead either to a difference in nestlings' diet and/or to a trade-off between organism's maintenance and sexual characters. These findings suggest that farming practices at a local scale surrounding nest locations may have subtle effects on chick development, but also on trade-offs between important physiological functions. This study highlights the importance of a multi-trait approach when assessing adverse and beneficial effects of both OF and CF on individuals.

1. Introduction

The biodiversity crisis, faced nowadays, spares no species. Among the most affected taxa, bird numbers decreased by 25% in Europe between 1980 and 2016 with farmland birds showing the steepest decline (Heldbjerg et al., 2018; Reif and Hanzelka, 2020; Rigal et al., 2023). At the same time, agricultural practices changed to increase yields, leading ecologists to hypothesise a causal link between agricultural intensification and farmland bird decline (Fuller et al., 1995; Krebs et al., 1999; Chamberlain et al., 2000). Among the features of this intensification, mechanisation appeared to have a direct negative impact on ground-nesting birds (Crick et al., 1994; Tews et al., 2013; Santangeli et al., 2015). Landscape homogenisation and the use of pesticides also raised concerns because they can have insidious effects on farmland species (Matson et al., 1997; Benton et al., 2003; Mason et al., 2013; Sumasgutner et al., 2019; Moreau et al., 2022a). To counter the negative impacts of conventional farming (CF) involving intensive agricultural practices, organic farming (OF) emerged as an alternative in the late 1970s (Lockeretz, 2007). OF is defined as a production system excluding synthetic pesticides and fertilisers that uses nature-based solutions (Keesstra et al., 2018) to promote and enhance agro-ecosystem health (Lockeretz, 2007).

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Three main interrelated characteristics distinguish OF from CF: agricultural practices, natural and semi-natural habitat proportion and farm inputs. Agricultural practices can be harmful to farmland birds either directly or indirectly. A direct impact of agricultural machines used under intensive practices is the death of ground-nesting birds (Crick et al., 1994; Vickery et al., 2001; Tews et al., 2013). Incubating adults, broods and chicks are frequently destroyed during mechanical mowing, tilling and harvesting activities (Schekkerman et al., 2009; Santangeli et al., 2015; Bretagnolle et al., 2018a). Agricultural practices can also have indirect effects on birds, by increasing disturbance and decreasing available resources. For instance, manure spreading, manual and mechanical weed and pest control, tillage and multiple cultivations all together generally increase labour in OF systems (Karlen et al., 1995; Jansen, 2000; Orsini et al., 2018), and can be identified as a potential source of disturbance for farmland birds. Human presence and activity are known to affect bird feeding rates (Exposito-Granados et al., 2020), induce physiological stress (Mougeot and Arroyo, 2017; Rabdeau et al., 2023), and alter behaviour including parental care (Fernández and Azkona, 1993), sometimes leading to nest failures (Strasser and Heath, 2013). Farming activity has even been shown to increase nest abandonment in passerine birds and to cause up to 32% nest failure in river lapwing, *Vanellus duvaucelii* (Assandri et al., 2017; Mishra et al., 2020). Moreover, intensive agricultural practices that involve clearing of ditches, removal of hedgerows, and intensification of grassland management are associated with lower territory and breeding densities in yellowhammer, *Emberiza citrinella* (Bradbury et al., 2000) and barn owl, *Tyto alba* (de Bruijn, 1994). These intensive land-uses commonly found in CF lead to simplified landscapes where important habitats for birds are no longer available (Bradbury et al., 2000; Sumasgutner et al., 2019; Tarjuelo et al., 2020) and where their food supplies are reduced through fewer weed seeds and natural insect reservoirs (Matson et al., 1997; Chamberlain and Wilson, 2000; Benton et al., 2002). Insects rely on the availability of non-uniform patches of grass, weeds and hedgerows for food and shelter (Benton et al., 2002). These features are thus associated with higher insect diversity (Holland and Fahrig, 2000) and higher bird diversity and abundance (Chamberlain et al., 1999; Chamberlain and Wilson, 2000; Freemark and Kirk, 2001). Hedgerows and landscape complexity also maintain small mammal populations (Butet et al., 2006; Gelling et al., 2007) and consequently have a positive effect on populations of birds of prey (Koks et al., 2007; Butet et al., 2010), even if some find such a link only under favourable food conditions (Sumasgutner et al., 2019). OF has been shown to have a positive effect on biodiversity, an effect which is even greater in simplified landscapes (Roschewitz et al., 2005; Fischer et al., 2011; Henckel et al., 2015). In fact, OF practices that might imply non-cropped habitat management such as the establishment of hedgerows and field margins benefit insects and therefore insectivorous birds (Hole et al., 2005; Lockeretz, 2007). Under OF agricultural systems, natural and semi-natural areas are typically higher than under CF systems (Gibson et al., 2007; Norton et al., 2009), hence OF promotes resources for birds, especially food abundance (Fischer et al., 2011). Different meta-analyses support that view, showing that OF promotes higher overall biodiversity, from flora to insects and bird species richness and abundance (Tuck et al., 2014; Stein-Bachinger et al., 2021). Lastly, farm inputs are the main characteristic contrasting OF from CF. Although organic plant protection products can be used, no synthetic pesticides or fertilisers are employed for crop production in OF systems. Indeed, in Europe for farms to be considered organic the prohibition of use of synthetic pesticides and fertilisers is the only mandatory practice, other practices such as natural or semi-natural areas management being generally recommended but not systematic in OF systems (Regulation EU, 2018/848 of the European parliament and of the Council of 30 May, 2018). Recent reviews highlighted that synthetic pesticides are the main factor negatively affecting farmland birds (Stanton et al., 2018; Rigal et al., 2023). Pesticides can have indirect effects on birds through the elimination of crop pests and weeds, reducing food and habitat resources (Hole et al., 2005; Gibbons et al., 2014). Furthermore, pesticides lack specificity and can affect non-target species (Sánchez-Bayo and Goka, 2014; Vyas, 2017). The use of pesticides can thus have direct effects on birds, either inducing lethal effects (immediate death after exposure) or delayed sublethal effects altering life history traits. In fact, different functions of a bird's organism can be affected by pesticides, from the cellular level to higher physiological functions and behaviours (Mitra et al., 2011; Moreau et al., 2021, 2022a; Gaffard et al., 2022). Many experimental studies have revealed altered life history traits due to pesticide toxicity, which may compromise bird survival, and consequently populations dynamics (Lopez-Antia et al., 2015a, 2015b; Ortiz-Santaliestra et al., 2020; see Moreau et al., 2022a for a recent review). *In natura*, birds are exposed to numerous molecules as multiple pesticides are usually applied to fields either at the same time or sequentially, inducing cocktail effects (Gaffard et al., 2022). Moreover, the persistence of some molecules in soil and water can lead to contamination without direct contact with the applied chemicals (Jagannath et al., 2008; Goulson, 2013; Fuentes et al., 2023). A recent study on wild passerines showed that birds nesting in hedges surrounded by CF were less vigorous than those nesting in hedges surrounded by OF, and that this might be due to pesticide contamination under CF (Moreau et al., 2022b).

Montagu's harriers, *Circus pygargus*, a migratory raptor species inhabiting agricultural lands in Europe, nest on the ground in cereal crops. In France, populations have declined by 10–30% over the past 30 years (Millon and Bretagnolle, 2004; Comolet-Tirman et al., 2015) and the principal cause is believed to be agricultural intensification (Butet and Leroux, 2001; Arroyo et al., 2002; Arroyo et al., 2004; Santangeli et al., 2015). Montagu's harriers mostly feed on common voles, *Microtus arvalis*, which display cyclic population dynamics in Western Europe, hence food availability and diet vary from year to year (Millon and Bretagnolle, 2008). In years of low vole abundance, Montagu's harriers compensate by feeding on Orthopterans (grasshoppers and crickets) (Salamolard et al., 2000). For this farmland specialist bird, the type of agricultural system at a local scale might be of great importance during the breeding season. Indeed, eggs and chicks are located on the ground in crop fields, and the altricial chicks stay in the nest during the whole rearing period (~35 days). Consequently, at all developmental stages, Montagu's harrier offspring might be affected by the composition and management of the habitats in the close surroundings. If the agricultural system in the vicinity leads to altered life history traits, survival may be compromised, reducing future recruitment of fledglings into the population of this declining species.

In the present study, we explored the patterns of life history traits variation in nestlings along a gradient of OF near their nests. Accordingly, we investigated the relative impacts of OF vs. CF on multiple life history traits of Montagu's harrier chicks at a local scale (up to 2000 m) around nests. Compared with OF, CF may decrease food availability (due to both lower quality habitats and intensive management practices) and increase exposure to pesticides (through contact or prey consumption). Therefore, we hypothesised that a higher percentage of CF around the nest may adversely affect fledglings. Since all life history traits involve complex interconnected functions, expectations for a single trait are complicated to raise. A multi-trait approach is therefore crucial to elucidate the effects of agricultural systems (OF vs. CF) on birds, and eventually to highlight potential trade-offs between life history traits. We expect chicks reared in nests surrounded by a lower percentage of OF (i.e., a higher percentage of CF) would have a poorer overall health status that might be reflected by inferior body condition, more markers (physical and biochemical) of chronic stress, a depressed immune system, and lower vigour than chicks surrounded by a higher percentage of OF.

2. Material and methods

2.1. Ethics statement

All experiments conformed to the French guidelines for the ethical use of animals in research (APAFIS#18557–2019010822312199v2). Bird handling was allowed and licensed by the Centre de Recherches sur la Biologie des Populations d'Oiseaux (CRBPO; Museum National d'Histoire Naturelle; licence #1308).

2.2. Study area and model species

The study site is located in southwestern France (46°11′N, 0°28′W), in the Long-Term Social-Ecological Research 'Zone Atelier Plaine & Val de Sèvre' (LTSER ZAPVS), a 450 km^2 area where annual crop identification is carried out, allowing mapping of organic crop plots in detail. In France, OF is regulated by the European legislation (Regulation EU, 2018/848 of the European parliament and of the Council of 30 May, 2018) on organic production, which details all practices to respect for farms to be considered organic, the main one being the complete ban on the use of synthetic pesticides and fertilizers. Other farming practices, including weeding or non-cropped habitat management, being at the discretion of each farmer. Nonetheless, Catarino et al. (2019) report general information on CF and OF systems of the study area; the main differences being smaller farm areas, more diversified crops and a lower nitrogen supply in OF systems. In this intensive agricultural area, cereal crops are the predominant cultures (average coverage of 41.5% between 2009 and 2016), followed by corn, sunflowers and oilseed rape (Bretagnolle et al., 2018b). Natural (or semi-natural) habitats and urbanized areas represent only a small proportion of the study area (average coverage of 13.5% and 9.8% respectively between 2009 and 2016; Bretagnolle et al., 2018b) thus only organic crop plots were used for analyses (see Section 2.7 for further information). Montagu's harrier monitoring has taken place in this site since 1994 (Bretagnolle et al., 2018b). This species nests on the ground, mainly in cereal crops, and lays up to eight eggs (Arroyo et al., 1998; Millon et al., 2008). The incubation period lasts 29 days and the rearing period between 30 and 35 days (Arroyo et al., 2007). The mean productivity in this site is 2.05 fledglings per breeding attempt (Arroyo et al., 2004), breeding success depending mainly on the availability of common voles, their main prey in this area (Salamolard et al., 2000). Foraging range varies with location; in the study area males use home ranges of $\sim$14 km^2 (Salamolard, 1997), but home ranges during the breeding season can stretch to 100 km^2 (Guixé and Arroyo, 2011).

2.3. Overall experimental design

From 2016 to 2021, the reproduction of Montagu's harriers was monitored from late April until late July. All nests were searched by professional ornithologists, and once located, visited and mapped using a global positioning system (GPS) receiver, they were stored on a geographical information system (GIS; QUANTUMGIS 3.16.5; QGIS Development Team, 2021, see Fig. 1). Nests were visited twice during the incubation period and every week after hatching (lag time between visits 7 ± 2 days). At 15 days old, chicks were sexed and banded with a unique coded aluminium ring from the Museum National d'Histoire Naturelle. Sex was determined by the colour of the iris (brown for females and grey for males) (Leroux and Bretagnolle, 1996).

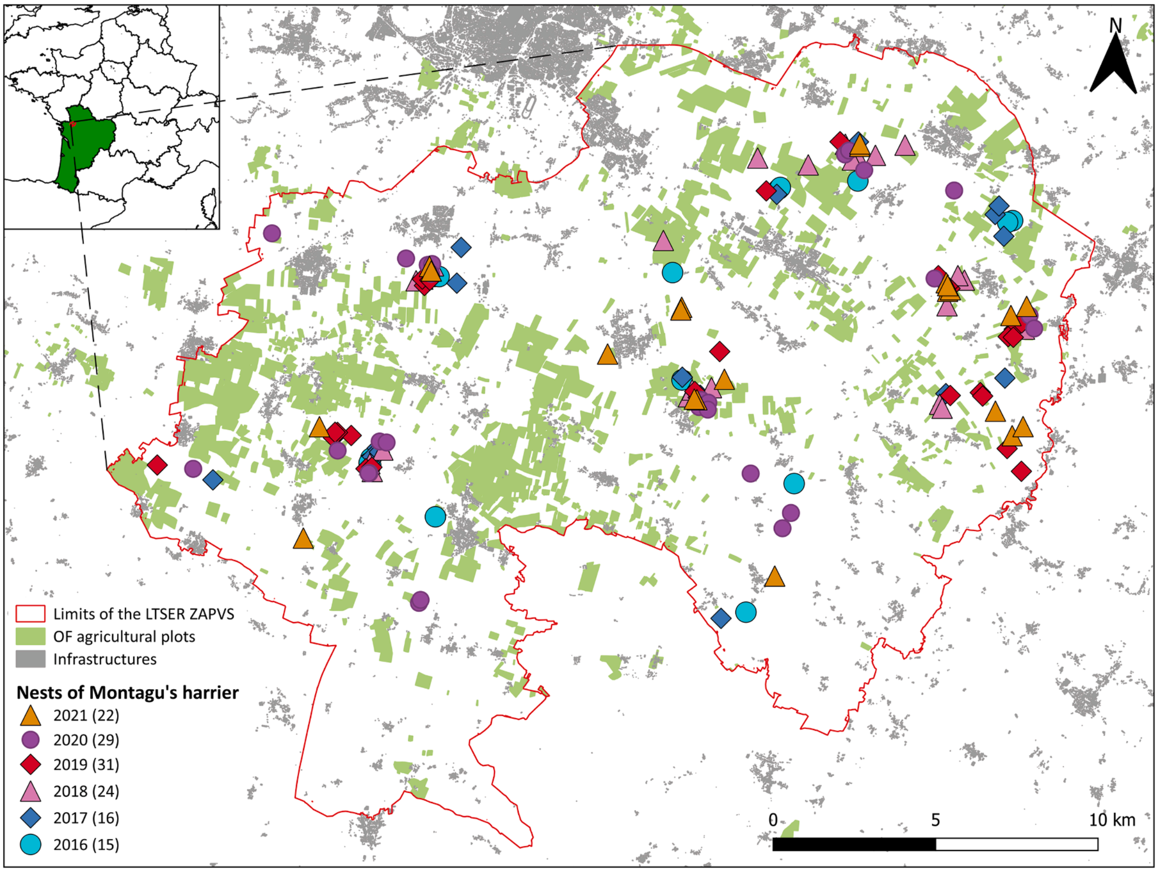

Fig. 1. Spatial distribution of Montagu's harrier, *Circus pygargus*, nests across the Long-Term Socio-Ecological Research Zone Atelier Plaine & Val de Sèvres (LTSER ZAPVS). Only nests corresponding to data for fledglings included in the present study are shown (the number of nests monitored each year is given in brackets). Organic farming (OF) agricultural plots correspond to data available for 2021 from our GIS database. Infrastructures correspond to buildings, sport fields, cemeteries, locks and bridges present in the study area.

For the present study, we focused on data collected during the last visit before fledgling, when nestlings were 26 ± 2 days old. Briefly (see details below), chicks were caught in the nest and carefully handled to take morphometric measurements and breath rate and collect blood samples (Fig. 2). A phytohemagglutinin (PHA) skin injection was performed, and behaviour during handling was also recorded (Fig. 2). Chicks were handled and released at the nest, and the handling time was minimised to reduce potential stress. The following day of the visit, nests were again visited to record chicks' inflammatory response to the PHA skin injection (see details below). A total of 380 chicks were monitored ($N_{2016} = 40$, $N_{2017} = 32$, $N_{2018} = 81$, $N_{2019} = 84$, $N_{2020} = 85$, $N_{2021} = 58$) from 137 nests ($N_{2016} = 15$, $N_{2017} = 16$, $N_{2018} = 24$, $N_{2019} = 31$, $N_{2020} = 29$, $N_{2021} = 22$). No sex ratio bias was observed for the entire period (187 females and 193 males; Binomial test $p = 0.80$).

2.4. Morphometric measurements

2.4.1. Morphometrics for body condition assessment

Body condition is a morphometric estimate reflecting the overall health status of individuals, notably involved in post-fledgling survival of birds (Blums et al., 2005; Vitz and Rodewald, 2011; Duijns et al., 2017). Body condition for each nestling was estimated from the scale mass index following Peig and Green (2009):

$$SMI_i = Mass_i \times \left(\frac{Mean \quad tarsus \quad length \quad of \quad the \quad group}{Mean \quad tarsus \quad length_i}\right)^{b_{sma}}$$

where b_{sma} is the slope of the major axis regression of log (body mass) on log (mean of the two measures of the left and right tarsus length) with the standard major axis method (package 'smatr') (Warton et al., 2012) for each chick *i*. The mass was obtained by weighing chicks with a Pesola 500 g spring scale (accuracy ± 5 g) (Schindellegi, Switzerland) and the left and right tarsus were measured twice with a digital calliper (accuracy ± 0.1 mm).

2.4.2. Fault bars

Fault bars are malformations in feathers induced by punctual stressors during chick growth, which may result in feather and even rachis breaks (Jovani and Rohwer, 2016; see Rabdeau et al., 2023 for an example of recent usage). Chick tails were photographed and the number of fault bars (all breaks in barbs and barbules) was counted on the most affected rectrix feather by the same experimenter to avoid observer bias. The number of fault bars on each chick ranged between 0 and 10.

2.4.3. Colouration

Colouration of nestling bare parts, a carotenoid-related trait, can be limited through the diet and mediated by different physiological effects (Garcia-Heras et al., 2017). The colour of the cere and tarsus (bare parts) were scored by direct comparison with a colorimetric chart from Yolk Colour Fan (Roche, Neuilly-sur-Seine, France). Scores ranged from 0 (almost white / pale yellow) to 15 (orange) and were highly correlated with colour parameters obtained using a spectroradiometer (Faivre et al., 2001). Cere scores ranged from 0 to 5 and tarsi ranged from 0 to 6. Both scores were highly correlated (Spearman's rank correlation test, $\rho > 0.77$, $p < 0.0001$, 95% confidence interval CI = [0.72, 0.81], n = 380), and only cere scores were used for statistical analyses.

2.5. Physiological measures

2.5.1. Breath rate score

Breath rate usually refers to a proxy of physiological stress (Carere and van Oers, 2004; Torné-Noguera et al., 2014; Rabdeau et al., 2019). Breath rate was measured using chicks handled in back position, and corresponded to the number of thoracic movements counted during 15 s for three consecutive replicates. The breath rate score was then obtained by averaging the three replicates.

2.5.2. Physiological parameters assessed from blood

2.5.2.1. Blood collection. Blood sampling was made on the brachial vein through a puncture using a sterile needle and heparinised capillaries. A single microcapillary of 10 μL was used for both sedimentation rate and haematocrit, a droplet of blood was used for blood smear (see below), and two capillaries of 100 μL each were placed in Eppendorf tubes and kept refrigerated (0–5 °C) for 2–3 h while transporting to the lab, where they were stored at − 20 °C until further analyses (carotenoid titration and acetylcholinesterase activity; see below).

2.5.2.2. Sedimentation rate and haematocrit. Erythrocyte sedimentation rate (ESR) corresponds to the proportion of red blood cells sedimented per hour, and it increases in response to various inflammatory or infectious processes (Saino and Møller, 1996). Microcapillaries were kept vertical for 5 h in a refrigerated container (0–5 °C), lengths were measured with an electronic calliper to the nearest 0.1 mm, and ESR in millimetres per h (mm/h) was estimated as follows:

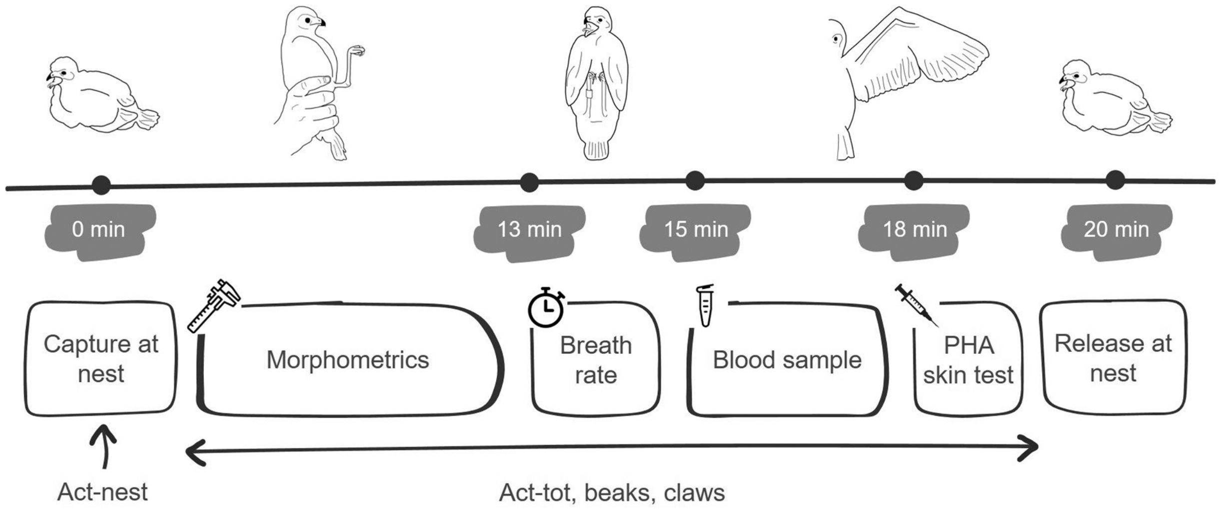

Fig. 2. Summary of the experimental design measuring different morphological, physiological and behavioural traits of Montagu's harrier, *Circus pygargus*, chicks before fledging. For each chick, act-nest = activity at nest approach, act-tot = total activity during handling, and beaks and claws = the rates of beak and claw attacks during handling.

$$ESR = \frac{volume\ not\ filled\ by\ erythrocytes}{total\ blood\ volume\ in\ the\ tube \times time\ of\ sedimentation}$$

Once ESR was measured, microcapillary tubes were centrifuged at 5000 rpm for 5 min to measure haematocrit. Haematocrit is used as an index of anaemia and corresponds to the proportion of the tube filled with erythrocytes divided by the total volume of blood sample in the tube, given as a percentage (Saino and Møller, 1996; see also Biard et al., 2015; Moreau et al., 2021).

2.5.2.3. Carotenoid titration. Carotenoids are involved in different functions of birds, including as antioxidants, and a high concentration in blood can reflect physiological stress (McGraw and Ardia, 2003). Eppendorf tubes containing ~150 μL of blood were centrifuged (10 min at 9000 rpm) and plasma was collected and kept at − 20 °C until laboratory analyses. Plasma carotenoid concentration was subsequently obtained in micrograms per millilitre (μg/mL) using a standardised colorimetric technique (see Biard et al., 2010 for additional information).

2.5.2.4. Acetylcholinesterase activity. Acetylcholinesterase (AChE) activity is negatively affected by some neurotoxic pesticide molecules altering neural functions, which in turn has disruptive consequences on diverse behaviours and complex functions (Grue et al., 1997; Eng et al., 2017; Moreau et al., 2022a). AChE activity in milliunits per minute (mU/min) was obtained by a method derived from Ellman et al. (1961) using 5 μL of blood diluted in 100 μL of a 1% Triton X-100 solution and measured using a SpectraMax iD3 microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA) over 40 min at 324 nm and 37 °C. AChE activity was calculated using SoftMax Pro7 software (Molecular Devices, San Jose, CA, USA) as the maximum change in absorbance per minute during kinetics analysis.

2.5.2.5. Blood smear. For each bird, a blood smear was performed with a droplet of blood sample. Slides were air-dried and fixed in absolute methanol before being stained with Giemsa solution (Sigma Chemical, St. Louis, MO, USA). Blood smears were examined with an oil immersion microscope under 1000x magnification. Total white blood cells (WBC) count was estimated from the number of leucocytes found among 8000 erythrocytes. A high number of leucocytes reflects stress and inflammatory processes (Ots et al., 1998). Different types of leucocytes were counted and used to estimate the relative proportion of heterophils to lymphocytes (H/L ratio). The H/L ratio is used as an index of physiological stress, as it is supposed to increase in response to various stressors (Maxwell, 1993; Ots et al., 1998; Moreau et al., 2021; see Biard et al., 2015 for details).

2.5.3. Immune challenge

Before release into the nest, the immune system of nestlings was challenged using phytohemagglutinin (PHA) skin tests (Smits et al., 1999). Briefly, 0.5 ± 0.1 mg of PHA (Sigma Chemical, St. Louis, MO, USA) diluted into 0.1 mL of phosphate-buffered saline (PBS) was injected subcutaneously in the wing patagium as described by Sternalski et al. (2012a). The thickness of the patagium was measured three times before injection using a pressure-sensitive dial thickness gauge (spessimeter accuracy ± 0.01 mm) and again 24 h after injection. The response to the PHA skin test corresponds to the change in average thickness (in mm) of the injection site, and thus the magnitude of this change reflects the immune potential of nestlings (Sternalski et al., 2012a; see also Biard et al., 2015).

2.6. Behavioural measures

Behaviour is considered an indicator of individual health that can be altered by multiple chemicals and is often regarded as a more sensitive response to stress or toxicity levels than other physiological measures (Grue et al., 1997; Clotfelter et al., 2004; Hellou, 2011; Moreau et al., 2022a). When handling chicks (i.e., from capture in the nest to release), the behaviour of bird was scored using four behavioural traits as described by Rabdeau et al. (2019): (i) activity during nest approach (act-nest); (ii) total activity during handling (act-tot); (iii) rate of beak attacks (beak); (iv) rate of claw attacks (claws). Act-nest was assessed when the experimenter approached the nest to catch the chicks and scored as follows: stayed motionless = 0, chicks retreated = 1, chicks ran away = 2. Act-tot was measured throughout handling and scored as follows: chicks stayed motionless = 0, chicks rarely moved = 1, chicks moved regularly = 2, chicks moved continuously = 3. The number of beak and claw attacks were counted during handling and divided by the duration of handling, resulting in a rate (number of attacks per minute).

2.7. Statistical analyses

A principal component analyses (PCA) was performed on the four behavioural scores using singular value decomposition of a standardised (centred and scaled) data matrix (Crawley, 2012). The first axis of the PCA reflecting global activity and vigour during handling (PC1-activity) was retained as its eigenvalue was > 1, and it accounted for 48.69% of the variance. PC1-activity was positively correlated with both rates of beak ($r = 0.56$) and claw ($r = 0.57$) attacks, act-tot ($r = 0.55$) and act-nest ($r = 0.24$) scores.

Normality and homoscedasticity of all response variables were tested using the ‘fitdistrplus’ package prior to model implementation, and when these assumptions were not met, distribution with the best fit was selected using the same package (Delignette-Muller and Dutang, 2015). Model residuals and spatial autocorrelation (using Moran’s I) were also checked using ‘DHARMa’ package (Hartig, 2022). PC1-activity, body condition, ESR, breath rate score, AChE activity and PHA response were used as response variables in Linear Mixed-effects Models (LMMs). Carotenoid concentration was square-root transformed, haematocrit and H/L ratio were log-transformed prior to LMM execution, since the distributions of these variables did not meet normality. Generalised LMMs fitted with a Poisson distribution (PGLMMs) were implemented for fault bars and colouration scores and a Generalised LMM fitted with a Negative Binomial distribution (NB-GLMM) was used for the total WBC count. For all models (LMMs, PGLMMs and NB-GLMM), the percentage of OF around the nest (log-transformed, hereafter OF effect) and the year as factor were included as fixed effects, and the nest identity as a random effect (to avoid pseudo-replication as chicks can belong to the same nest) (Bolker et al., 2009). The year was considered a fixed effect because we wanted to control for inter-annual variability of environmental conditions (phenology, food availability and weather for instance). Because year could have been considered a random factor, we ran all models changing only the year fixed effect into a random effect. This approach did not change the results obtained with year as a fixed effect, hence we used the results of the first analyses. All models were run with the ‘lme4′ package (Bates et al., 2015).

As we had no a priori information on the scale of the OF effect on life history traits for each chick, we estimated the percentage OF around the nest for 20 buffer sizes (i.e., radii every 100 m, from 100 m to 2000 m). To this end, the GPS coordinates of nests and a database of the LTSER ZAPVS, which georeferenced the agricultural plots using OF practices, were used with the ‘sf’ package (Pebesma, 2018). Since OF agricultural plots varied over time, the percentage of OF around nests for a given year was calculated using the georeferenced plots of the corresponding year. We ran 20 models per life history trait (one per buffer size) and extracted an Akaike Information Criterion corrected for small sample size (AICc) using the ‘AICcmodavg’ package (Mazerolle, 2020). From a set of candidate models, AICc allows selection of the model with the most support, which has the lowest AICc, assuming that models with $\Delta AICc < 2$ (i.e., a difference in AICc between candidate models <2) are similarly supported and cannot be distinguished from each other (Burnham and Anderson, 2004). For every life history trait, a plot was generated representing the AICc of the 20 models (one for each buffer size), with a red dotted line illustrating the model selection criterion (lowest AICc of the candidate models + 2), with the shape and colour reflecting the significance and direction of the OF effect. To avoid type II errors, we chose to present our results based on effect sizes (estimates of the model) assorted with their 95% confidence intervals (CI) (following Garamszegi, 2006; Nakagawa, 2004; Nakagawa and Cuthill, 2007; Cumming, 2014). The OF effect direction corresponded to the sign of the estimate and was considered significant when 95% CI did not include 0. Calculation of the bootstrapped CI was obtained for 2000 iterations and using the package 'boot' (Canty and Ripley, 2022). We discuss effect sizes assorted with their 95% CI which allow to quantify the biological effect we tested and provide comparable estimates for future research and meta-analyses, still, we provide the results from model comparison using likelihood ratio-based χ^2-statistics in supplementary materials (Fox and Weisberg, 2011).

To assess potential links between variables, Spearman's rank correlation tests were performed. All statistics were conducted with R v4.0.5 software (R Core Team, 2021), implemented using the aforementioned packages.

3. Results

Summary statistics and Spearman's rank correlation coefficients of the 12 life history traits are presented in the Supplementary Material (see Table S1 and Fig. S1). Only ESR and haematocrit (Spearman's rank correlation test, $\rho > -0.42$, $p < 0.0001$, 95% CI = [−0.51; −0.32], n = 343) were correlated (Supplementary Material Fig. S1). The set of candidate models for body condition, fault bars, breath rate score, ESR, haematocrit, carotenoid concentration, AChE activity, WBC count, and PHA skin test did not yield any specific buffer size with substantial support (i.e., AICc <2 with a significant OF effect) for an influence of OF percentage around nests (Fig. S2 in supplementary materials). For cere colouration score and H/L ratio, the best models (i.e., those with ΔAICc <2) identified a positive effect for OF percentage at a range of radii (Fig. 3). The cere colouration score increased with OF percentage around the nest for buffer sizes > 500 m (Fig. 3a). For H/L ratio, a positive relationship was detected between 300 and 900 m (Fig. 3b). Concerning the PC1-activity, a positive relationship with the percentage of OF was detected at 200 m around the nests (Fig. 3c).

We then selected the model with the lowest AICc to probe the relationships between the trait and the OF percentage around the nest (Fig. 4; Chi-squared test results are given in Table S2 in supplementary materials). Chicks displayed a significantly higher cere colouration score when surrounded by a higher percentage of OF at 1500 m (effect size = 0.14; 95% CI = [0.04; 0.22]), the lowest AICc for this life history trait (Fig. 4a). The lowest AICc for the H/L ratio was at a radius of 600 m around nests; at this distance the H/L ratio significantly increased with increasing OF percentage (effect size = 0.11; 95% CI = [0.04; 0.18]; Fig. 4b). For PC1-activity, the lowest AICc was at 200 m, an increase in OF at this distance from nests significantly increased the global activity of chicks (effect size = 0.20; 95% CI = [0.06; 0.40]; Fig. 4c).

4. Discussion

The aim of the present study was to explore life history traits variation of Montagu's harrier chicks linked to agricultural systems around nests. The results revealed an effect on two of the 12 traits investigated, although moderate. Cere colouration and H/L ratio were positively linked to OF at different radii, suggesting that the effects of the agricultural system operate at different distances, but not necessarily at the same range. The cere colouration score increased with increasing OF percentage at distances > 500 m with the best model selected at 1500 m. The H/L ratio increased significantly with OF at distances from 300 to 900 m, the best model being at 600 m. Global activity of chicks increased with increasing OF percentage at 200 m only. For all other life history traits, namely body condition, fault bars, breath rate score, ESR, haematocrit, carotenoid concentration, AChE activity, WBC count, and PHA skin test, no effect of OF was detected at any of the buffer sizes.

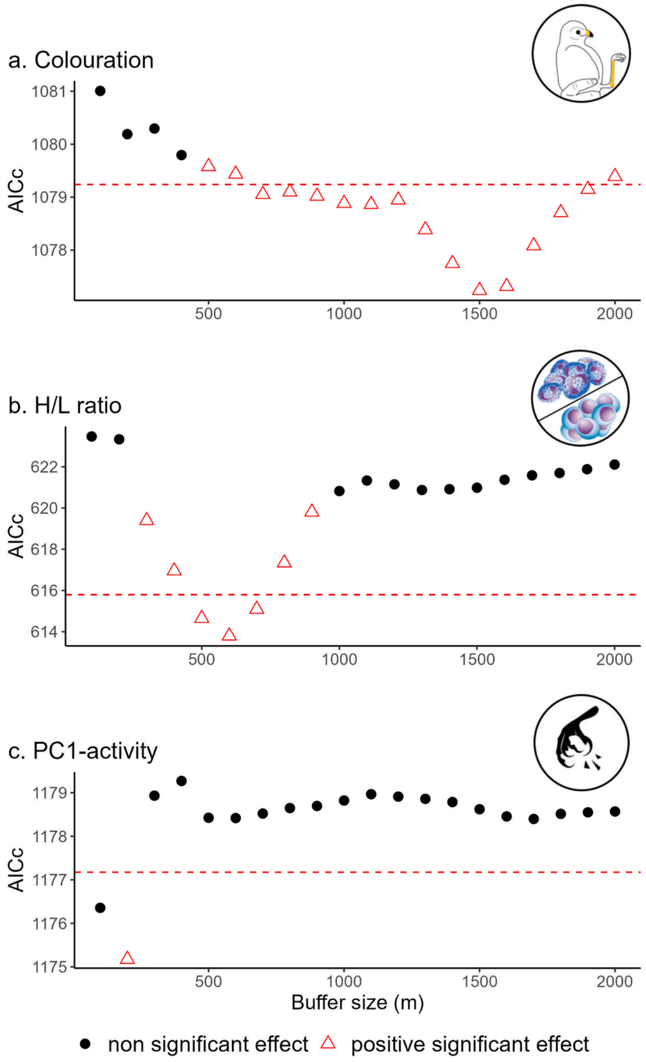

Fig. 3. Model selection using AICc to assess the responses of life history traits (life history traits) of Montagu's harrier, *Circus pygargus*, chicks to the effect of the percentage of organic farming (OF) around the nest at different buffer sizes (from 100 m to 2000 m radius). Measured life history traits are (a) cere colouration score, (b) H/L ratio, and (c) PC1-activity. AICc = Akaike Information Criterion corrected for small sample sizes. Red dotted lines correspond to the lowest AICc of candidate models + 2. Red triangles indicate a positive significant OF effect (i.e., a positive model estimate and assorted 95% confidence interval (CI) not crossing 0). Black dots represent a non-significant OF effect (i.e., the 95% CI of model estimate crosses 0).

In the present study, we found evidence for the impacts of farming practices (CF and OF) on Montagu's harrier chicks at a low spatial scale (< 2000 m) around nests. First, OF was found to be associated with a higher H/L ratio. This trait is indicative of stress undergone by chicks during their rearing period. Indeed, H/L ratio, is considered a reliable indicator of mild to moderate stress, and to reflect chronic stress levels rather than short-term stress responses (Maxwell, 1993; O'Dell et al., 2014). This result suggests that chicks in areas with a higher OF percentage may suffer more disturbance. Indeed, compared with CF, OF practices involve more repetitive passages (Karlen et al., 1995; Reissig et al., 2016; Orsini et al., 2018), which may equate to more stress experienced by chicks. Furthermore, areas with more OF fields are generally better appreciated than areas with CF fields by urban

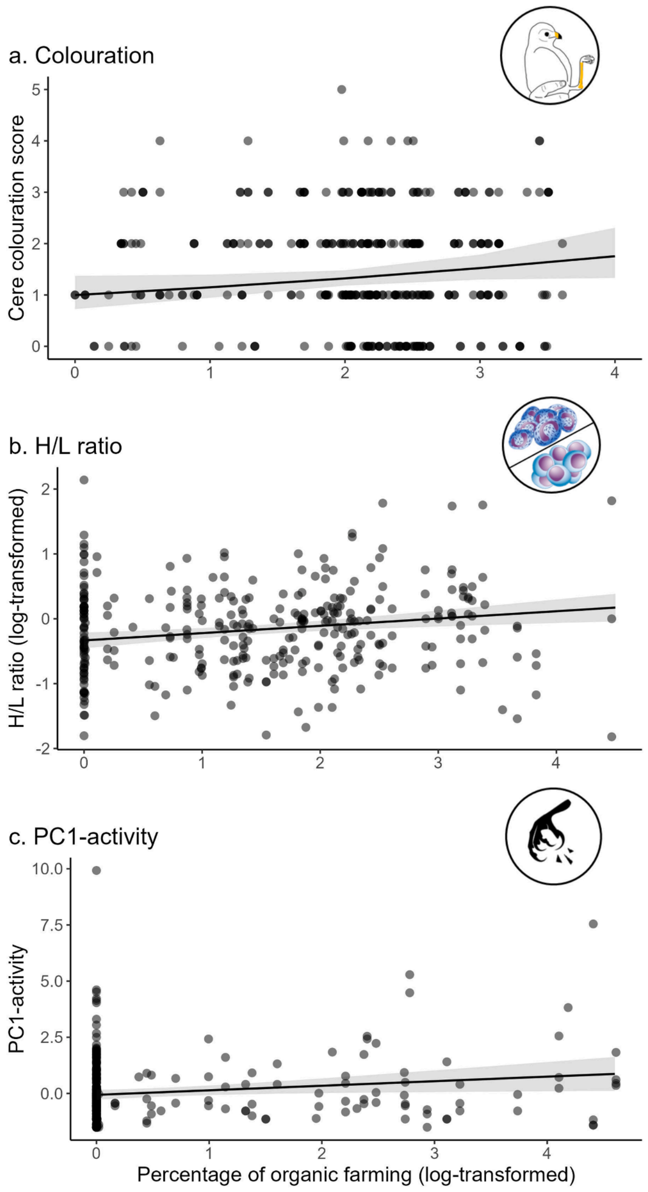

Fig. 4. Organic farming effect on (a) cere colouration score, (b) H/L ratio, and (c) PC1-activity of chicks at 1500 m, 600 m and 200 m around the nest respectively. A dot represents a value measured for a chick (darker = more chicks had this value). Lines predict values of each life history trait depending on the percentage of organic farming. Shading represents the 95% confidence interval.

inhabitants attracted to naturalistic features and elements (Zasada, 2011). Diversified habitats offer more opportunities for recreation and leisure than uniform ones (Hansen et al., 2006). Anthropic disturbances can induce stress in Montagu's harrier chicks, leading to physiological responses to this stress and even to the formation of fault bars (see Rabdeau et al., 2023). Repeated human activities, whether related to OF practices or recreation, could also induce prolonged absence of adults, resulting in reduced parental care, and thus more stress. Additionally, a higher presence of conspecifics or other raptor species around OF crops is also possible. Competition between Montagu's harriers nesting in semi-colonies is presumably low but could increase in years of low vole abundance. The presence of other raptor species foraging in the same area would also increase competition for food (Garcia and Arroyo, 2002). Competition for food resources might increase the time parents spend foraging, leaving chicks alone for longer periods. The presence of more raptors may increase the risk of predation of Montagu's harrier nests, which are particularly vulnerable and attractive in low vole years (Arroyo et al., 2001). Pairs are often seen mobbing common buzzards, *Buteo buteo*, or black kites, *Milvus migrans,* to protect their nests (authors' personal observations), and this might result in more frequent parental absences. Consequently, an increase in intra- and interspecific negative interactions surrounding OF crop plots would increase the time chicks are unattended.

An alternative explanation for the higher H/L ratio observed for chicks in nests surrounded by a high OF percentage is the presence of a higher number and/or diversity of pathogens. Indeed, H/L ratio is known to increase in response to infectious diseases as well as psychological disturbances (Ots et al., 1998), hence we cannot exclude the potential effect of increased pathogen exposure in OF systems. Pesticides may reduce pathogen diversity in CF systems compared with areas in which OF is more prevalent (Goutte and Molbert, 2022). Therefore, chicks from nests located in these zones might be more prone to parasite exposure, which could, in turn, explain why they exhibit a higher number of heterophil leucocytes, and thus a higher H/L ratio. Moreover, several studies reported that H/L ratio is species-specific, and that it may vary between captive and wild individuals, depending on sex, age, nutritional and infectious status, and even brood size (Ruiz et al., 2002; Limiñana et al., 2009; Ortego et al., 2009; Parejo and Silva, 2009; Banbura et al., 2013; Catitti et al., 2022). Previous studies on Montagu's harriers attempted to set reference interval values for haematological parameters of captive adults and free-living nestlings, but reliable comparisons were difficult to make with the present work, and further investigation is required (Lavin et al., 1993; Limiñana et al., 2009). Additionally, even if chicks in OF and CF areas face similar parasite diversity, their investment in different life history traits might vary. For instance, chicks from nests mainly surrounded by CF may face a trade-off between investment for immune parameters and other life history traits such as growth (Sheldon and Verhulst, 1996; Norris and Evans, 2000). A recent study on grey partridge showed that hatchlings exposed to pesticides were of lower body size compared with those not exposed, but they underwent compensatory growth to compensate for the initial differences (Gaffard et al., 2022). In the present work, we did not observe any difference in body condition, but growth trajectories should be properly investigated to exclude this hypothesis.

At a local scale, a high percentage of CF is presumed to reduce available food resources; carotenoids being provided by the diet, this may explain why the colouration of the cere, a sexual trait indicative of chick quality (Sternalski et al., 2012b), is lower for chicks surrounded by CF compared with chicks surrounded by OF. Still, we did not detect a difference of carotenoid concentrations in chicks' plasma. Nevertheless, even if CF and OF provide an equivalent amount of food resources, it is possible that OF provides a greater diversity of food supply. Indeed, as mentioned above, OF promotes insect and passerine bird diversity, both of which are alternative prey for Montagu's harriers. Nestling colouration mainly depends on the proportion of insects and birds in their diet since these are richer in carotenoids (Sternalski et al., 2010). Thus, it is likely that chicks surrounded by OF benefit from higher carotenoid levels and/or richer carotenoid composition in their diet, resulting in more intense colouration of secondary sexual traits. In fact, carotenoid composition rather than carotenoid level in food items are responsible for colouration differences in some bird species (Eeva et al., 2010), thus studying carotenoids composition in more details using chromatographic methods would help to investigate this. Besides, various studies highlighted the covariation between colouration and immunocompetence in bird nestlings, due to the role of carotenoids in both immune functions and colouration (Saino et al., 2000, 2003; Baeta et al., 2008). Thus, competing demand for carotenoids for colouration and the immune system might occur. However, we did not detect any effect of the agricultural system on the immune responses of nestlings assessed by PHA skin tests at a local scale, while the colouration of the cere was lower in chicks mainly surrounded by CF. Another potential trade-off leading to lower colouration scores in chicks surrounded by CF is the allocation of carotenoids to detoxification processes (Edge et al., 1997; Krinsky, 1998; see Bustnes et al., 2007). Indeed, exposure of birds to pollutants is known to induce oxidative stress, leading to a higher demand for antioxidants (including carotenoids) for redox processes (Ortiz-Santaliestra et al., 2015; Moreau et al., 2021, reviewed in Moreau et al., 2022a). Some studies on wild raptors found compensatory responses between different functions, linked to low concentrations of pesticides altering bird health (Bustnes et al., 2004; Rivera-Rodríguez and Rodríguez-Estrella, 2011; Ortiz-Santaliestra et al., 2015; Garcia-Heras et al., 2018). Despite the absence of a significant effect of the agricultural system on plasma carotenoid concentrations in the present work, we can suppose that pesticides used in CF might affect carotenoid allocation in Montagu's harrier nestlings, leading to trade-offs involving other physiological functions such as detoxification and oxidative stress, not measured here. A proper titration of pesticides in chicks' blood would be needed to rule about this trade-off.

The global activity level of chicks has been found to be higher when the surrounding OF at 200 m increases. This result matches with previous findings on 6 passerine birds (Moreau et al., 2022b), which reported a higher vigour of individuals when they originated from organic hedgerows compared to those captured in conventional ones. The main hypothesis raised by the authors is a lower exposition of birds to pesticides in OF systems and not an increased food abundance as birds did not show variation in body condition (Moreau et al., 2022b). In our case, the same pattern arises as we did not detect an effect of OF on body condition of Montagu's harrier chicks. Still, as we detected a beneficial effect of OF on chicks' activity only at 200 m around nests, the potential role of pesticides in their behavioural differences is questionable. Moreover, we did not detect an effect of agriculture systems on the AChE activity which, until proven otherwise, discard a neurotoxic effect of pesticides on chicks here. The increased chick activity when there is more OF at 200 m could be alternatively explained by human disturbances. In fact, after crops harvests, nests are exposed to curious walkers who can visit nests untimely, generating aggressive reactions of chicks and eventually sensitization to humans (Rabdeau et al., 2019, 2023). Besides, just as for the H/L ratio, increased labour and/or recreation in OF fields compared to CF fields could lead to an even greater exposure of chicks to humans. However, even if the effect size seems to indicate a medium effect of OF on the vitality of chicks, the assorted CI is relatively large suggestive of a large variability among chicks (Nakagawa and Cuthill, 2007; Cumming, 2014), interpretation on this result should thus be considered with caution.

5. Conclusion

In the current context of overall biodiversity loss, it is crucial to understand the relative impacts of CF and OF practices which are often opposed one to the other. However, as evidenced by our results, the picture is quite more complex than just black and white. The multi-trait approach used here revealed that CF practices can have detrimental effects on chick quality, and that OF can also exert adverse effects on their physiological stress. In fact, studying only the colouration of secondary sexual traits would have led to partial conclusions about the relative effects of CF on chick quality, whereas studying additionally morphometric and physiological traits that are indicative of stressors, such as H/L ratio, facilitated complete conclusions. In fact, both agricultural systems may impact Montagu's harrier chicks, with potential consequences for immediate survival during migration and later during adulthood. Multi-trait approaches are important because population dynamics reflect complex physiological processes of individuals that shape life history traits (development, reproduction, and survival), which in turn impact populations.

However, the present study is preliminary, as based on a rough cost/benefit analysis of these agricultural systems. One may also consider that we did not expect large effect size for the impact of agricultural systems on chicks since the relationships among the different life history traits and how they are shaped are quite complex. For example, this study did not consider that chicks are fed by their father, who may hunt up to several kilometres away, potentially masking/compensating for low habitat quality at the local scale (Guixé and Arroyo, 2011; Krupiński et al., 2020). One way to consider the influence of the diet would be to analyse the composition of pellets through observational and morphological analyses or even using metabarcoding from buccal and/or cloacal swabs (Nota et al., 2019; Brouellette, 2021). Our study only considered the habitats in the vicinity of the nest from a binary point of view (OF vs. CF), and therefore did not capture all parameters such as the proportion of natural, semi-natural or urban habitats in the landscape potentially influencing chick health. Human activities related to OF and CF should be investigated in more detail to further determine the effects of different types and frequencies of disturbances on ground-nesting farmland birds. Finally, OF and CF contrast in their use of pesticides, which may partly explain some of our results (see Moreau et al., 2022a for an overview), especially knowing that pesticide use is the main driver of birds' decline in Europe (Rigal et al., 2023). Therefore, in future work, titration of pesticides in blood should be performed to explore their impact on chick health.

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Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2023.108719.

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Supplementary material

Fig. S1. Spearman's rank correlation coefficients for all life history traits of Montagu's harrier chicks analysed in this study (380 chicks, 137 nests, all years included). Dot colour represents the direction of the correlation (purple = positive, orange-brown = negative) and dot size is proportional to effect size (larger dots = larger effects).

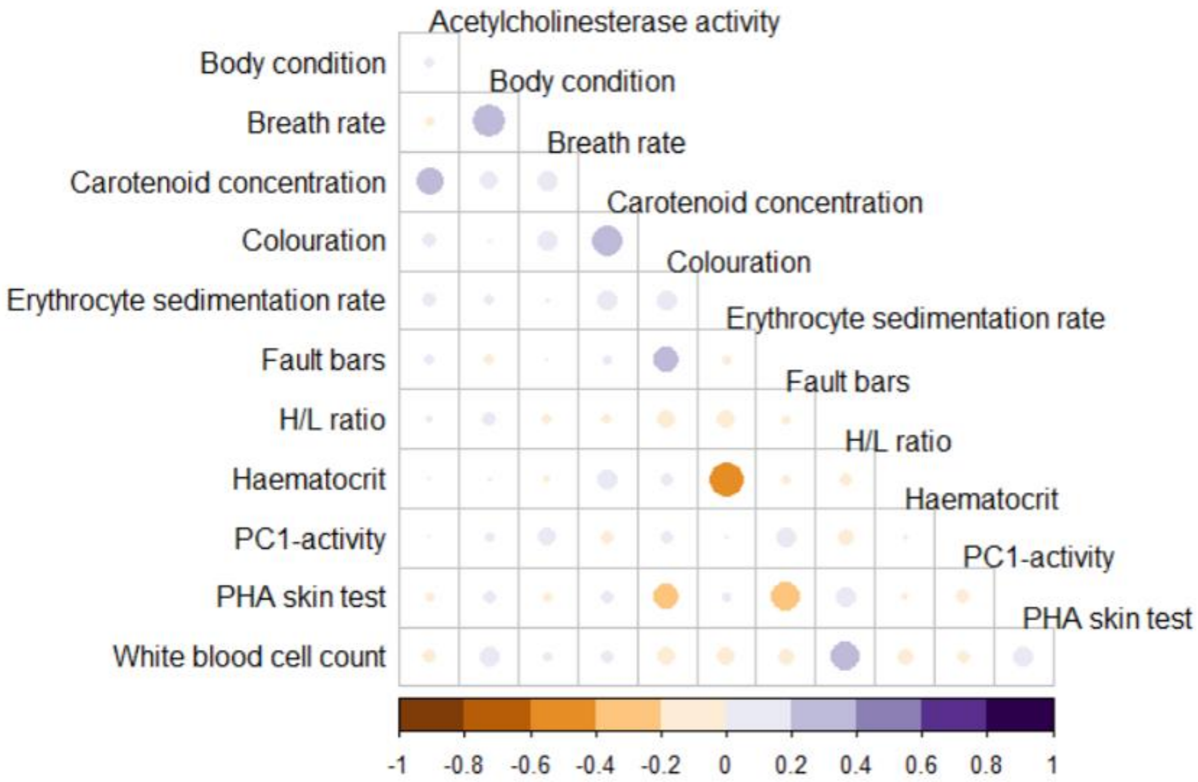

Fig. S2. Model selection using AICc to assess the responses of life history traits (LHTs) of Montagu's harrier, *Circus pygargus*, chicks to the effect of the percentage of organic farming (OF) around the nest at different buffer sizes (from 100 m to 2000 m radius). Measured LHTs are (a) body condition, (b) fault bar, (c) breath rate, (d) erythrocyte sedimentation rate (ESR), (e) haematocrit, (f) carotenoid concentration, (g) acetylcholinesterase activity, (h) white blood cell count and (i) PHA skin test. AICc = Akaike Information Criterion corrected for small sample sizes. Red dotted lines correspond to the lowest AICc of candidate models + 2. Black dots indicate a non-significant OF effect (i.e., the 95% confidence interval of model estimate crosses 0).

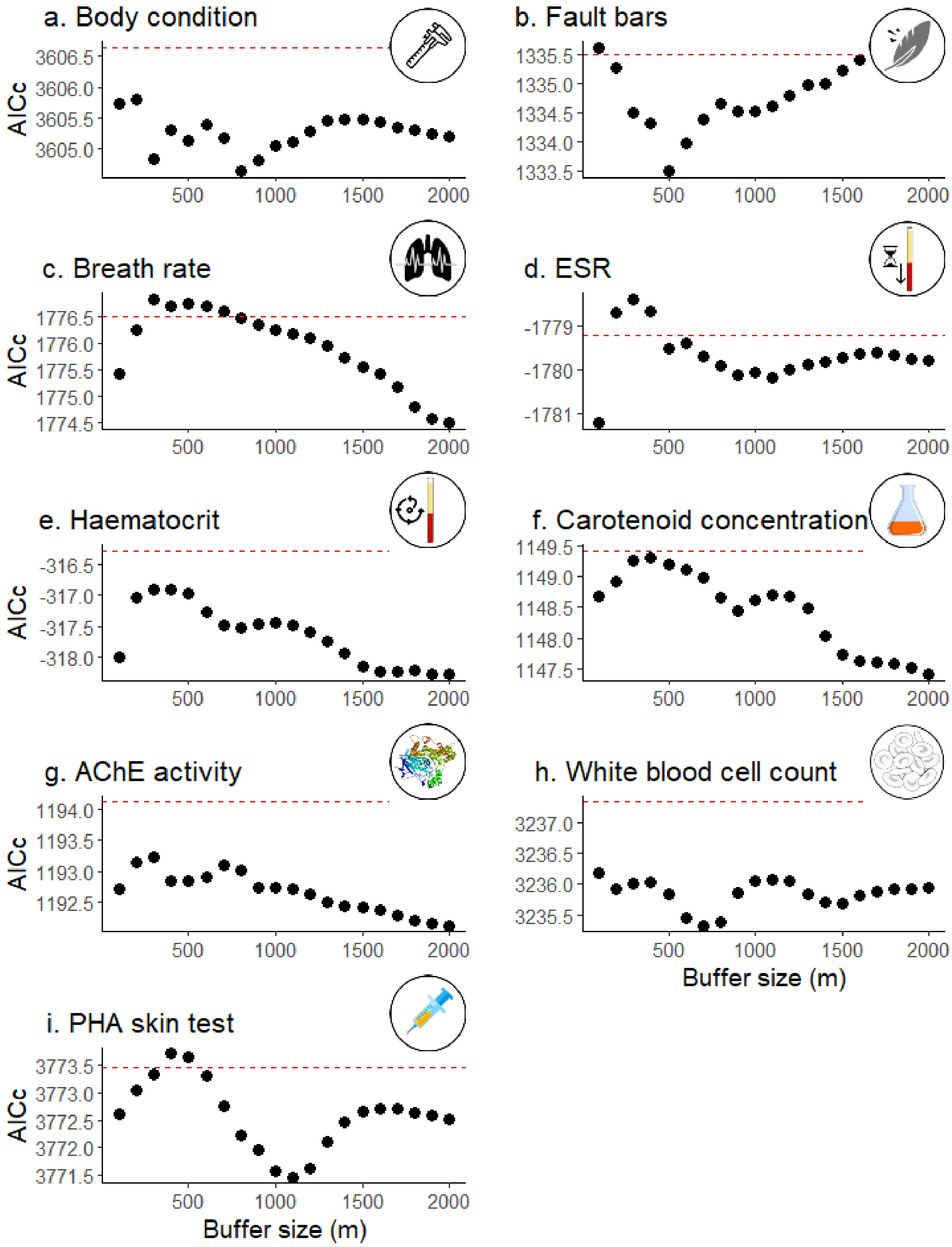

Table S1. Summary statistics for 12 life history traits (LHTs) measured for 380 Montagu's harrier chicks from 137 nests monitored between 2016 and 2021. Descriptive statistics given are N = sample size (number of chicks), mean, SD = standard deviation, and min-max = minimum and maximum values. Units of measure next to the corresponding LHT are g = grams, mm/h = millimetres per hour, % = percentage, µg/mL = micrograms per millilitre, mU/min = milliunits per minute, and mm = millimetres.

Measured life history traits (units)	2016		2017		2018		2019		2020		2021	
	N	**Mean ± SD** (min-max)	N	**Mean ± SD** (min-max)	N	**Mean ± SD** (min-max)	N	**Mean ± SD** (min-max)	N	**Mean ± SD** (min-max)	N	**Mean ± SD** (min-max)
Morphometrics												
Body condition (g)	40	**316.0 ± 25.1** (253.9 - 372.4)	31	**313.2 ± 29.8** (248.7 - 399.3)	81	**312.0 ± 30.3** (244.6 - 395.1)	84	**309.0 ± 32.2** (224.1 - 430.0)	85	**330.5 ± 30.0** (272.7 - 403.0)	57	**336.3 ± 34.8** (274.8 – 447.5)
Fault bars	37	**3.6 ± 1.8** (1 - 8)	25	**3.1 ± 1.3** (1 - 6)	81	**4.5 ± 2.0** (1 - 10)	69	**2.8 ± 1.6** (0 - 8)	84	**2.1 ± 1.6** (0 - 8)	53	**3.0 ± 1.6** (0 - 8)
Cere colouration	40	**1.6 ± 1.3** (0 - 5)	32	**1.6 ± 0.8** (0 - 3)	81	**2.2 ± 0.9** (0 - 4)	84	**0.9 ± 0.9** (0 - 4)	85	**1.1 ± 1.0** (0 - 3)	58	**1.8 ± 1.0** (0 - 4)
Physiology												
Breath rate score	0	NA	32	**15.05 ± 3.21** (8.00 - 21.67)	81	**15.89 ± 3.53** (9.67 - 27.00)	84	**14.80 ± 3.60** (8.67 - 32.67)	84	**16.07 ± 3.36** (9.00 - 24.33)	58	**15.88 ± 2.89** (9.00 - 21.67)
Erythrocyte sedimentation rate (mm/h)	35	**0.04 ± 0.02** (0.01 - 0.10)	30	**0.07 ± 0.02** (0.04 - 0.12)	79	**0.08 ± 0.02** (0.03 - 0.13)	77	**0.06 ± 0.02** (0.02 - 0.13)	84	**0.07 ± 0.02** (0.02 - 0.15)	57	**0.07 ± 0.02** (0.03 - 0.12)
Haematocrit (%)	26	**36.86 ± 5.49** (30.36 - 58.97)	31	**38.63 ± 6.45** (23.29 - 49.69)	79	**37.10 ± 5.40** (27.26 - 50.25)	77	**37.74 ± 5.73** (23.61 - 62.98)	83	**38.10 ± 4.46** (25.21 - 50.42)	52	**40.41 ± 7.22** (31.96 - 62.84)
Carotenoid concentration (µg/mL)	37	**18.36 ± 15.28** (0.68 - 60.20)	27	**20.60 ± 15.50** (2.02 - 57.61)	70	**25.17 ± 15.91** (1.82 - 118.20)	52	**16.91 ± 9.06** (0.28 - 37.13)	66	**25.81 ± 23.16** (2.91 - 113.70)	55	**45.91 ± 21.76** (14.66 - 102.82)

Acetylcholinesterase activity (mU/min)	0	NA	0	NA	74	**14.09 ± 1.66** (10.55 - 18.12)	69	**12.66 ± 2.16** (5.02 - 17.26)	79	**14.34 ± 2.20** (7.34 - 20.21)	49	**16.16 ± 2.99** (9.58 - 22.76)
White blood cell count	22	**96.86 ± 52.77** (10 - 211)	30	**68.83 ± 34.67** (25 - 174)	62	**71.92 ± 31.04** (23 - 188)	81	**101.12 ± 30.94** (29 - 163)	80	**94.61 ± 46.39** (32 - 398)	51	**86.69 ± 36.38** (22 - 188)
H/L ratio	21	**2.26 ± 1.77** (0.50 - 8.50)	30	**1.25 ± 1.10** (0.36 - 5.94)	61	**0.73 ± 0.82** (0.16 - 6.17)	81	**1.12 ± 0.77** (0.25 - 5.78)	80	**0.98 ± 0.73** (0.19 - 5.68)	51	**0.93 ± 0.44** (0.25 - 2.19)
PHA skin test (mm)	39	**81.1 ± 63.7** (-6.0 - 227.0)	30	**109.8 ± 52.9** (10.0 - 264.7)	81	**99.2 ± 41.6** (0 - 239.0)	76	**153.6 ± 50.6** (52.0 - 279.3)	74	**157.2 ± 54.6** (21.7 - 288.3)	57	**132.4 ± 52.8** (28.7 - 276.7)
Behaviour												
PC1_activity	0	NA	32	**0.19 ± 1.21** (-1.50 - 4.22)	81	**0.22 ± 1.55** (-1.50 - 7.54)	81	**-0.06 ± 1.63** (-1.50 - 9.92)	84	**-0.33 ± 0.96** (-1.50 - 2.68)	58	**0.15 ± 1.38** (-1.50 - 5.29)

Table S2. Effects of organic farming (OF) percentage around nests and year on four life history traits of Montagu's harrier, *Circus pygargus*, nestlings. Tests were performed using Linear Mixed-effects Models (LMMs) for H/L ratio (log-transformed) and PC1-activity, and Generalised LMMs fitted with a Poisson distribution (PGLMMs) for colouration score (see text for details). Significant effects are in bold. Sample sizes are indicated below each life history trait.

Response variables *(sample size)*	Fixed factors	Chi-squared test		
Colouration score *(380 chicks, 137 nests)*	OF	$\chi^2 = 4.030$	df = 1	***p* < 0.05**
	Year	$\chi^2 = 44.812$	df = 5	***p* <0.001**
H/L ratio (log-transformed) *(324 chicks, 128 nests)*	OF	$\chi^2 = 11.159$	df = 1	***p* < 0.001**
	Year	$\chi^2 = 67.991$	df = 5	***p* <0.001**
PC1-activity *(336 chicks, 122 nests)*	OF	$\chi^2 = 4.963$	df = 1	***p* < 0.05**
	Year	$\chi^2 = 4.644$	df = 4	*p* = 0.326

Chapter II

Pesticide contamination patterns and factors of influence

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Summary of Chapter II

The heavy use of synthetic inputs in conventional agriculture has led to the ubiquitous presence of pesticides in agroecosystems. These substances have been found in a wide range of abiotic and biotic compartments, including soils, invertebrates, small mammals and birds. However, before my research work, few studies biomonitored a large range of pesticides, especially through non-destructive methods, and only one study to my knowledge did so using Montagu's harrier nestlings' blood. This study screened 90 compounds of herbicides, fungicides, insecticides and metabolites in 29 nestlings from Germany, detecting only two pesticides in a few contaminated chicks. Therefore, there was first a need to describe how farmland birds from an intensive farming area are contaminated with pesticides: what substances, what prevalence and in what concentrations. This knowledge is crucial to assess the actual level of contamination with pesticide mixtures of farmland birds and therefore to estimate if there is a risk from pesticide use on them. The analytical method developed to search and quantify 104 different substances in the blood matrix, including herbicides, fungicides, insecticides, a synergist and a safener, allowed me to describe the contamination patterns of Montagu's harrier nestlings sampled in 2021 (*ARTICLE 2*). Among the 55 chicks monitored in 22 nests, 28 different substances were detected including 10 herbicides, 12 fungicides, 5 insecticides, and 1 synergist. The mixtures found in nestlings were mainly composed of herbicides, with some nestlings being contaminated by the three types of pesticides. Given the ecology of the species, the patterns of agricultural pesticide use in the study area and the substances detected in nestlings' blood, Montagu's harrier nestlings were proposed as valuable biosentinels for pesticide biomonitoring schemes. Moreover, as no one ever investigated how this contamination to pesticide cocktails could vary within and between nestlings, and which factors could be of influence, there was a need to provide initial responses. A better understanding of the factors that could influence pesticide cocktails could help set relevant biomonitoring schemes and eventually find means to reduce farmland bird's exposure to pesticides. I therefore applied a first approach that consisted of the use of metrics and investigation of factors largely used/investigated in ecotoxicological studies, although these concerned heavy metals and POPs, including legacy organochlorine insecticides. Two metrics were thus used to assess pesticide contamination levels in Montagu's harrier chicks: the total number of substances detected and the sum of their concentrations. All nestlings sampled in 2021 had at least one substance detected in their blood with a minimal sum of concentrations of 24 pg.mg^{-1}, and a maximum of 16 substances and 7266 pg.mg^{-1}. Because there seemed to be a high inter and intra-brood variability in pesticide contamination levels, I first assessed if this variability could influence the sampling design of biomonitoring schemes (i.e., sampling randomly one nestling per nest or the older nestling or all of them for instance) (*ARTICLE 3*). I found that nestlings originating from the same nest were as different in their contamination levels as nestlings from two different nests. Consequently, the biomonitoring of pesticide mixtures should consider the sampling of all nestlings in a nest to provide reliable estimates of the pesticide contamination within the agroecosystem. Besides, following the methodology applied in the first chapter, I considered the influence of organic farming (OF) fields around nests on contamination levels of Montagu's harrier chicks and found that increasing the proportion of OF at 300 m around nests decreased the number of pesticides detected in their blood. This result emphasised the role of OF as a conservational tool that reduces the exposure of farmland birds to pesticide cocktails and their associated effects. Other factors that could influence nestlings' contamination levels, notably their sex and hatching rank, were also investigated. None of these factors influenced pesticide contamination levels of nestlings, suggesting that the intra-brood variability may originate from a differential food intake of siblings (*ARTICLE 3*). Indeed, brood competition may lead to unequal ingestion of food items and thus of pesticides and nutrients/antioxidants that may ultimately result in contrasted contamination levels among siblings. The influence of the age and body condition of nestlings and the sampling date were further investigated on 35 nestlings sampled from 22 nests monitored in 2018 (*ARTICLE 4*). These chicks were sampled twice during their rearing period so that their contamination levels were determined at two ages spanning from 10 to 29 days old. From the initial 104 substances, 12 additional compounds were optimised and validated so that 116 substances were searched in the 70 blood samples, among which nine herbicides, four fungicides and five insecticides were detected. The contamination levels of chicks ranged from two to nine pesticides and 145 to 2384 pg.mg^{-1} in the first samplings (chicks were aged between 10 and 23 days old) and from zero to nine pesticides and up to 3473 pg.mg^{-1} in the second samplings (between 23 and 29 days old). I found that these contamination levels were significantly reduced with the increasing age of chicks but were unrelated to their body condition or the sampling date. One of the possible mechanisms could be a greater metabolic and excretion capacity of older nestlings. Nonetheless, from the six pesticides detected in both samplings (seven were detected only in the first sampling and five in the second), only ethofumesate had an age-dependent decrease in concentrations while the others were unrelated to the investigated factors. These results shed light on the complexity of the temporal fate of pesticide mixtures within wild farmland birds and point out the greater risk for younger chicks exhibiting heavy pesticide loads. In this chapter, as in other studies (*articles X and XX in appendices*), the repeated detection of banned substances brought up questions about the contamination routes of farmland birds. While for recently banned molecules persistence is the most parsimonious explanation, for molecules banned for decades, the question of fraudulent use and/or maternal transfer may be raised. Therefore, the titration of pesticides in other environmental compartments of the study area, especially in food pellets and unhatched eggs collected at nests, would be needed. Moreover, now that this chapter revealed the pesticide mixtures in Montagu's harrier chicks, the following step would be to assess the potential cocktail effects on their development and life-history traits.

ARTICLE 2

PESTICIDE CONTAMINATION PATTERNS IN MONTAGU'S HARRIER (*CIRCUS PYGARGUS*) CHICKS

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NEW STRATEGIES AND NEW CHALLENGES FOR PESTICIDE STUDIES: HOW TO COMBINE THE PRESERVATION OF OUR ENVIRONMENT AND SUSTAINABLE AGRICULTURE?

Pesticide contamination patterns in Montagu's harrier (Circus pygargus) chicks

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Abstract

Biomonitoring of persistent pesticides in birds of prey has been carried out for decades, but few studies have investigated their relevance for the monitoring of non-persistent pesticides. Herein, we determined the contamination patterns of multiple pesticides in Montagu's harrier (*Circus pygargus*) chicks in an intensive farming area of southwestern France. Blood samples from 55 chicks belonging to 22 nests in 2021 were assessed for 104 compounds (herbicides, fungicides, insecticides, safeners and synergists). All chicks had at least one herbicide in their blood, and half had at least two compounds. The 28 compounds detected comprised 10 herbicides, 12 fungicides, 5 insecticides and 1 synergist. Mixtures in blood were predominantly composed of herbicides, and six chicks presented a mixture of the three pesticide classes. The most prevalent compounds were sulcotrione (96% of chicks), tebutam (44%) and chloridazon (31%), of which the latter two had been banned in France for 19 and 3 years, respectively, at the time of sampling. Most compounds are considered non-acutely toxic, but sulcotrione is potentially carcinogenic, mutagenic and reprotoxic, raising questions about the effects on the health of nestlings. Biomonitoring of multiple pesticides through Montagu's harrier chicks in agroecosystems is clearly relevant because it reflects the general pattern of agricultural pesticide use in the study area. It also raises questions about exposure pathways in chicks, and further investigations are needed to disentangle the roles of dietary routes and maternal transfer for the established pesticide contamination patterns.

Keywords Biomonitoring · Farmland bird · Fungicide · Herbicide · Insecticide · Multiresidue pesticide analysis

Introduction

Agricultural intensification in the 1950s led to the massive use of chemical inputs as fertilisers and pesticides (Chamberlain et al. 2000; Stanton et al. 2018). Pesticides are now found in all compartments of agroecosystems including soils, earthworms, bees, nectar, small mammals and birds, years after being banned in some cases (Wintermantel et al. 2020; Fritsch et al. 2022; Pelosi et al. 2022; Fuentes et al. 2023). These products are the main drivers of declining farmland bird populations and have been associated with human diseases (Xu et al. 2022; Rigal et al. 2023). In a *One*

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Health context, determining pesticide contamination levels in wild species could facilitate estimation of human exposure to such contaminants (García-Fernández et al. 2020). In this regard, biomonitoring, which consists in the monitoring of the quality of an environment through wildlife, is crucial. In fact, biomonitoring of contaminants using bird species has been carried out for decades (Newton et al. 1993; Becker et al. 1994; Dauwe et al. 2002; Bustnes et al. 2007). Although most research has focused on heavy metals and persistent pollutants in seabirds and raptors (Albert et al. 2019; Helander et al. 2008; Crosse et al. 2012), attention is now being paid to biomonitoring of non-persistent pesticides—those not belonging to persistent organic pollutants (POPs)—in birds (Humann-Guilleminot et al. 2019, 2021; Badry et al. 2022; Fuentes et al. 2023). Birds have multiple advantages that make them valuable candidates as bioindicators; their biology and ecology are well known; they occupy various positions in the food chain, and they are easier to sample using non-lethal methods than many other taxa that may need to be destroyed (Becker 2003; García-Fernández et al. 2020). Among the different sampling methods, feathers and deserted eggs have been widely used as they constitute non-invasive methods (Becker et al. 1994, 2003; Burger and Gochfeld 1997; Espín et al. 2016). However, feathers may reflect past contamination from another site because contaminant deposition into feathers occurs as they grow (Espín et al. 2016), and eggs only reflect contamination of part of a population, namely breeding females (Pacyna-Kuchta 2023). More recently, blood sampling has received much attention because it reflects short-term exposure to contaminants (Espín et al. 2016), addressing one of the drawbacks of using birds as bio-indicators: their high mobility that may not reflect local environmental contamination (Becker 2003). Moreover, since only a small amount of blood is required for analyses, it allows the sampling of any individual from a population throughout the year. Together, these characteristics make blood a highly efficient matrix for biomonitoring (Espín et al. 2016; Pacyna-Kuchta 2023).

Birds of prey are particularly interesting as bio-indicators as they occupy high positions in trophic chains, making them vulnerable to biomagnification of contaminants (DesGranges et al. 1998; Voorspoels et al. 2007). Their populations have suffered severe declines since the 1960s due to acute poisoning by rodenticides, as well as the effects of POPs such as DDT (Ratcliffe 1967; Furness et al. 1989; Newton and Wyllie 1992; Fremlin et al. 2020). Various studies have highlighted their potential for biomonitoring programmes at large scales, most notably in Europe (Gomez-Ramirez et al. 2014; Badry et al. 2020; González-Rubio et al. 2021). Montagu's harrier (*Circus pygargus*) is a valuable candidate for pesticide biomonitoring because this raptor is a specialist predator of agroecosystems. Individuals nest on the ground in cereal crops, exposing eggs and chicks to pesticides present in the culture. The chicks are altricial, which exposes them to contaminants through contact with the soil and crops, through the air and through the diet during the ~ 4 weeks they spend in the nest before fledging. Parents mainly bring common voles (*Microtus arvalis*) to their chicks, alongside orthopteran insects and passerine birds as alternative prey (Salamolard et al. 2000), which can themselves be contaminated with pesticides. Although adult Montagu's harriers may be exposed to pesticide contamination in their wintering areas, chicks are mostly naïve in terms of pesticide contamination, except in cases of maternal transfer of certain molecules since females may detoxify themselves through egg-laying (Mineau 1982). Even so, contamination patterns in chicks are expected to largely reflect exposure to pesticide contamination in their local environment. The aim of the present study was thus to investigate to what extent Montagu's harrier chicks in an intensive farming area were contaminated by pesticides through blood sampling to specifically reflect their recent contamination.

Materials and methods

Study area

The Long-Term Socio-Ecological Research Zone Atelier Plaine & Val de Sèvre (LTSER ZAPVS) is a 435 km^2 area of intensive farming located in southwestern France (46°11′N, 0°28′W). The ZAPVS landscape is mainly composed of winter cereal crops (41.5%) and other dominant crops including sunflower (10.4%), corn (9.6%) and oilseed rape (8.3%) based on average cover for 2009 – 2016 (Fig. 1) (Bretagnolle et al. 2018). Meadows and urban areas represent approximately 13.5% and 9.8% of the study area, respectively (Bretagnolle et al. 2018). Organic crop plots in the ZAPVS accounted for ~ 18% of the surface in 2021. Organic farming practices in the area comply with European legislation (Regulation EU 2018/848 of the European Parliament and of the Council of 30 May 2018).

Model species

Montagu's harriers have been monitored since 1994 in the ZAPVS. In this site, their mean productivity is 2.05 fledglings per breeding attempt, mainly depending on the availability of their main prey, common voles (Salamolard et al. 2000; Arroyo et al. 2004), although females can lay up to eight eggs (Arroyo et al. 1998). The incubation period lasts 29 days, and chicks hatched asynchronously are reared until they fledge at the age of 30 – 35 days (Arroyo et al. 2004). Males ensure food provisioning of incubating females and chicks, and females join in with provisioning later in the

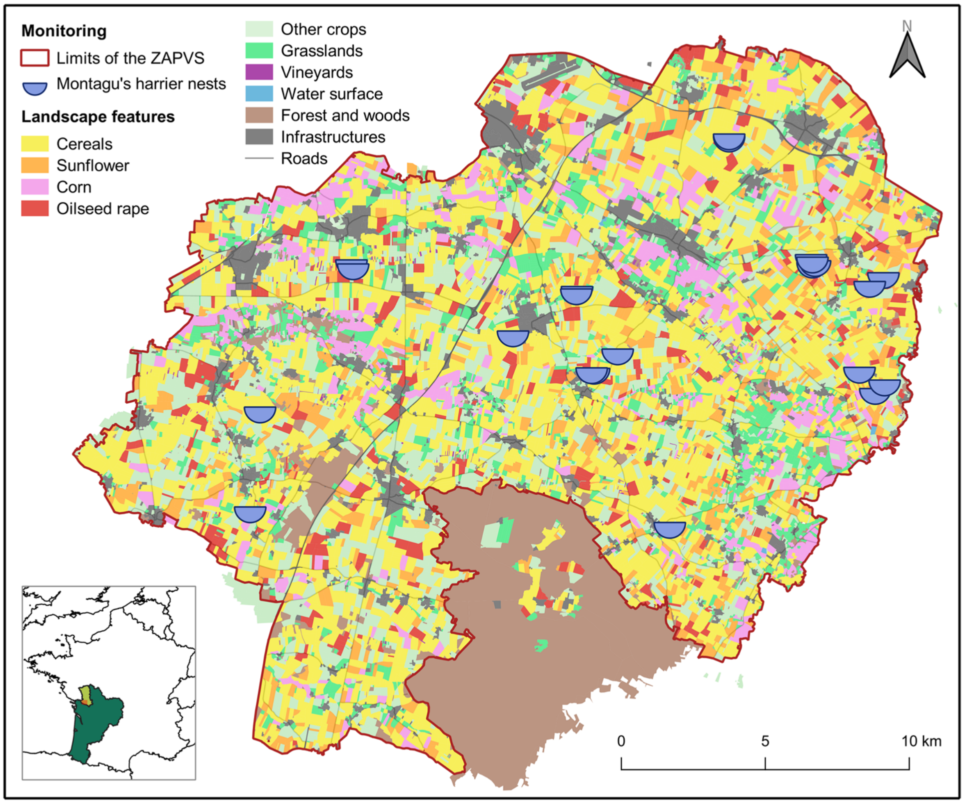

Fig. 1 Localisation of the 22 *Circus pygargus* nests monitored and landscape composition of the Zone Atelier Plaine & Val de Sèvres (ZAPVS) in 2021

rearing period (García and Arroyo 2005). Home ranges during the breeding season can stretch up to 100 km^2, although foraging ranges in the study area are generally ~ 14 km^2 (Salamolard 1997; Guixé and Arroyo 2011).

Sampling procedure

In 2021, professional ornithologists located and visited Montagu's harrier nests. The locations of nests were recorded on a geographical information system (GIS; QUANTUMGIS 3.22.16; QGIS Development Team 2023; Fig. 1) using coordinate data. Nests were visited twice before eggs hatched and every week after hatching. At 15 days old (second visit), chicks were banded with an aluminium ring with a unique code provided by the Museum National d'Histoire Naturelle de Paris (France) and sexed according to the colour of their iris, grey for males and brown for females (Leroux and Bretagnolle 1996). At 26 ± 2 days old (fourth visit), chicks were carefully caught and released at the nest to collect blood samples. From each chick, a 50 µL blood sample was collected in an Eppendorf tube by puncturing the brachial vein using a sterile needle and heparinised capillaries. Samples were placed in a cooler (0 – 5 °C), transported to the laboratory and stored at – 20 °C until further analyses. A total of 55 chicks from 22 nests were sampled between late June and early August 2021 with no sex ratio bias observed (26 males and 29 females; binomial test $p = 0.53$).

Multiresidue pesticide analysis

Whole blood samples (i.e., red blood cells and plasma) were analysed following the method developed by Rodrigues et al. (2023). Plant protection products (PPPs) are commonly referred to as pesticides, but they are composed of at least one active ingredient (herbicide, fungicide or insecticide) in a mixture with synergists (increasing the actions of pesticides) or safeners (improving herbicide selectivity towards weeds rather than crop plants). The analytical method employed allowed the detection and quantification of 104 compounds, mainly active molecules (i.e., pesticides) among the most used in France, including one synergist (piperonyl butoxide) and one safener (benoxacor). Hereafter, 'pesticide' refers to all compounds searched, including safeners/synergists.

Blood samples were thawed and weighed before extraction, and 10 µL of a carbendazim-d4 solution at 1 mg L^{-1} was added to each blood sample as an internal standard to monitor both the liquid–liquid extraction and purification steps. Carbendazim-d$_4$ was then quantified in each sample to ensure extraction recovery. For all samples, the average recovery for carbendazim-d4 was calculated, and no variation greater than 15% was observed, which was considered acceptable. Each sample was mixed with 2 mL dichloromethane and ethyl acetate (1:1) and homogenised by vortexing for 1 min followed by three rounds of sonication for 10 min each time. After each sonication, samples were centrifuged for 5 min, and supernatants were pooled and gently evaporated under a fume hood until reaching a final volume of 500 µL. The resulting extract was stored at − 20 °C until pesticide level analyses were conducted by liquid chromatography coupled to tandem mass spectrometry (LC/MSMS) and gas chromatography coupled to tandem mass spectrometry (GC/MSMS) using multiple reaction monitoring (MRM) for quantification. A blank and a standard were injected every ten blood samples to check for carryover and system contamination or variability.

For more volatile compounds, GC/MSMS analyses were conducted using an automatic thermal desorption system (ATD 350, PerkinElmer Corp., Norwalk, CT, USA) connected to a Trace 1300 GC coupled to an ITQ 900 mass spectrometer (Thermo Scientific, Illkirch-Graffenstaden, France). Compounds desorbed by ATD were separated on a Macherey–Nagel OPTIMA XLB capillary column (30 m × 0.25 mm i.d; 0.25-µm film thickness) with helium as the carrier gas at a constant flow of 1.2 mL min^{-1}. Spectra were obtained in electron impact ionisation (EI) mode at an electron energy of 70 eV.

For less volatile pesticides, LC/MSMS analyses were performed using a TSQ Quantum Access Triple Quadrupole Mass Spectrometer (Thermo Scientific) in heated positive electrospray ionisation (HESI+) mode, coupled with a Thermo Accela 1250 pump and a Thermo Combi Pal autosampler (Thermo Scientific). A Nucleodur C18 Pyramid column (150 mm × 3 mm, 3 µm i.d.) was employed for gradient mode analyses using a mobile phase of water and acetonitrile, both containing 0.1% formic acid.

For both LC/MSMS and GC/MSMS, prior to injection, samples were dopped with an added mixture of internal standards (trifluralin-d$_{14}$, nitrophenol-d$_4$, 2,4-D-d$_3$ and pendimethalin-d$_5$) used for quantification to avoid any potential instrument variations. Concentrations were thus calculated using calibration from commercialised standards of analytical purity using an internal standard method. Calibration was performed in triplicate by spiking both a blank matrix and a non-matrix. Responses showed good linearity, with correlation coefficients of ≥ 0.98 using a linear regression model. The matrix effect was assessed by comparing the standard deviations of the slopes for calibrations with and without the matrix, and no variation greater than 15% was observed.

Multiresidue analysis involved the detection and quantification of 104 pesticide molecules in MRM detection mode for both instrumentations. For each analyte and internal standard, two MRM transitions were monitored for each target compound; the first product ion was used for quantitation, and the second product ion was used for qualification. The ion ratio was also monitored for all transitions. Limit of detection (LOD) and limit of quantification (LOQ) were determined as three and ten times the ratio of the average noise height on either side of a known amount of a compound's peak to the peak height, respectively. The aim was to establish the minimum peak height necessary to distinguish a compound's peak from surrounding noise. LODs and LOQs for all detected compounds are listed in Table 1. For further details concerning the analytical method, refer to Rodrigues et al. (2023).

Results

At least one herbicide was detected in all Montagu's harrier chicks, half had at least two compounds in their blood, and one nestling had 16 (Fig. 2). Twenty-eight different compounds were detected (concentrations > LOD) in blood samples: 10 herbicides, 12 fungicides, 5 insecticides and 1 synergist (piperonyl butoxide; Table 1). Of these, 26 were quantified (concentrations > LOQ). Ten of the pesticides detected were banned for sale and use before sampling in 2021, among which tebutam and flusilazole were banned > 10 years ago (Table 1). Fifteen of the 28 compounds were considered non-acutely toxic because their birds' oral 50% lethal dose (LD50 = quantity of pesticide killing 50% of test animals) was > 2000 mg kg^{-1}, below the level needed to place them in acute toxicity hazard categories according to EC Regulation No. 1272/2008 (Table 1 and Table S1 and S2 in Supplementary Information). Five substances—carbetamide, propyzamide, dimoxystrobin, sulcotrione and cyproconazole—were considered carcinogenic, mutagenic and reprotoxic based on the nomenclature including hazards to the aquatic environment and human health (Order of 22nd December 2022) (Table S2). The distribution of different classes of compounds (herbicides, fungicides, insecticides and synergists) among chicks is depicted in Fig. 3. Of the 55 nestlings, 36% had at least one fungicide, 18% had at least one insecticide and 9% had the synergist piperonyl butoxide. For 28 nestlings (50%), the mixture of pesticides was composed of only herbicides, and 40 (73%) had a mixture dominated by herbicides. Mixtures combining herbicides, fungicides and insecticides were found in six chicks, of which one also had piperonyl butoxide (Fig. 3a). In terms of concentrations in blood, the distribution of the four types of compounds varied but herbicides remained predominant for 50 nestlings (91%), while fungicides and insecticides exceeded half of the total concentration for three and one chicks, respectively (Fig. 3b). Occurrences, concentration ranges and means ± standard deviations for each compound are listed in Table 2. The most frequent compounds detected were three herbicides: sulcotrione, tebutam and chloridazon, in 53 (96%), 24 (44%) and 17 (31%) nestlings, respectively (Table 2). Sulcotrione had the highest concentrations in blood with a maximum of 3184.67 pg mg^{-1}.

Table 1 Main properties, analytical methods (GC = ATD-GC–MS/MS; LC = LC–MS/MS) and limit of detection (LOD) and quantification (LOQ) in pg mg^{-1} for the 28 compounds detected in the blood of Montagu's harrier (*Circus pygargus*) chicks. Compounds are ordered by pesticide type, then alphabetically. The ban corresponds to prohibition years in France obtained from legislative texts (https://www.legifrance.gouv.fr/) accessed on 16 November 2023. DT50 (detection time 50% = time to detect a 50% decrease in pesticide concentration) ranges were obtained from field studies (for more details, see Lewis et al. 2016). Model species correspond to birds for which the oral LD50 (lethal dose 50% = quantity of pesticide killing 50% of test animals) was obtained: *Colinus virginianus* (Cv), *Coturnix japonica* (Cj), *Anas platyrhynchos* (Ap) and *Serinus canaria* (Sc). Log *P* corresponds to the log of the partition coefficient and measures the lipophilicity of molecules (the larger the value, the more lipophilic). Main crops, DT50, bird LD50, model species and log *P* were compiled from the Pesticide Properties DataBase (PPDB) of the University of Hertfordshire (http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm) accessed on 16 November 2023 (Lewis et al. 2016). Main crops of application were indicated if present in our study area and in line with plant protection product (PPP) guidelines (https://ephy.anses.fr/; accessed 16 November 2023). *NA* not applicable when not considered an active substance of PPPs in Europe

Type, compound	Ban (year)	Main crops	DT50 range (days)	Bird LD50 (mg kg^{-1})	Model species	Log *P*	Method	LOD	LOQ
Herbicide									
Bifenox	No	Cereals	8.3 – 32.1	> 2000	Cv	3.64	GC	0.0012	0.0038
Carbetamide	No	Alfalfa, vegetables	8	> 2000	Cv	1.78	LC	0.0053	0.0176
Chloridazon	Yes (2018)	Beets	3 – 105	> 2000	Cv	1.19	GC	0.0213	0.0709
2,4-MCPA	No	Cereals, meadows, linseed	25	377	Cv	−0.81	GC	0.1579	0.5263
Mecoprop-P	No	Cereals	21	> 500	Ap	−0.19	GC	0.0500	0.1667
Metamitron	No	Beets	11.1	1302	Cj	0.85	GC	0.0577	0.1923
Oxadiazon	Yes (2018)	Grass	90 – 330	> 2150	Cv	5.33	GC	0.0086	0.0286
Propyzamide	No	Oilseed rape	13.9 – 271.3	6578	Cj	3.27	GC	0.0021	0.0071
Sulcotrione	No	Corn, linseed	10.8 – 89.7	> 1350	Ap	−1.7	LC	0.0021	0.0071
Tebutam	Yes (2002)	Oilseed rape	60	> 5000	Ap	3	GC	0.0526	0.1754
Fungicides									
Boscalid	No	Cereals, sunflowers, linseed, peas, vegetables, fruits, vineyards	196 – 312.2	> 2000	Cv	2.96	GC	0.0005	0.0016
Carbendazim	Yes (2014)	Cereals, sunflowers, peas, beets, vineyards, soybeans	20 – 40	> 2250	Cv	1.48	LC	0.0042	0.0140
Cyproconazole	No	Cereals, beets, grass, vineyards	62.1 – 501.2	94	Cv	3.09	GC	0.0192	0.0639
Cyprodinil	No	Cereals, fruits	11 – 98	> 500	Ap	4	GC	0.0011	0.0036
Difenoconazole	No	Cereals, corn, vegetables,	20 – 265	> 2150	Ap	4.36	GC	0.0359	0.1196
Dimethomorph	No	Fruits, vegetables, vineyards	34 – 54	> 2000	Cv	2.68	GC	0.0072	0.0242
Dimoxystrobin	No	Wheat, oilseed rape	2 – 39	> 2000	Cv	3.59	GC	0.0038	0.0128
Epoxiconazole	Yes (2019)	Cereals, beets	52 – 226	> 2000	Cv	3.3	LC	0.0027	0.0091
Flusilazole	Yes (2008)	Cereals, beets, oilseed rape, fruits	63 – 240	> 1590	Ap	3.87	GC	0.0144	0.0481
Myclobutanil	No	Grass, fruits, vineyards	9 – 66	510	Cv	2.89	GC	0.0214	0.0714
Prochloraz	No	Cereals, oilseed rape, fruits, grass	28.6 – 245	662	Cv	3.5	GC	0.0170	0.0568
Quinoxyfen	Yes (2019)	Cereals, grapes, cucurbits, tomato	13 – 190	> 2250	Cv	5.1	GC	0.0048	0.0161
Insecticides									
Bifenthrin	Yes (2019)	Ornamentals, sports fields, lawns	65 – 125	1800	Cv	6.6	GC	0.0035	0.0116
Clothianidin	Yes (2018)	Corn, sorghum, fruits	13.3 – 1386	430	Cv	0.90	LC	0.0103	0.0344
Cypermethrin	No	Cereals, oilseed rape, vegetables, beets, fruits, grassland	9.3 – 31.2	> 9520	Ap	5.55	GC	0.0013	0.0042
Indoxacarb	No	Corn, vegetables, fruits	4.9 – 7.5	73.5	Cv	4.65	GC	0.0069	0.0231

Table 1 (continued)

Type, compound	Ban (year)	Main crops	DT50 range (days)	Bird LD50 (mg kg^{-1})	Model species	Log *P*	Method	LOD	LOQ
Thiacloprid	Yes (2018)	Beets, corn, vegetables	5.95 – 16.8	35	Sc	1.26	LC	0.0014	0.0048
Synergist									
Piperonyl butoxide	NA	NA	13	> 2250	Cv	4.75	GC	0.0004	0.0015

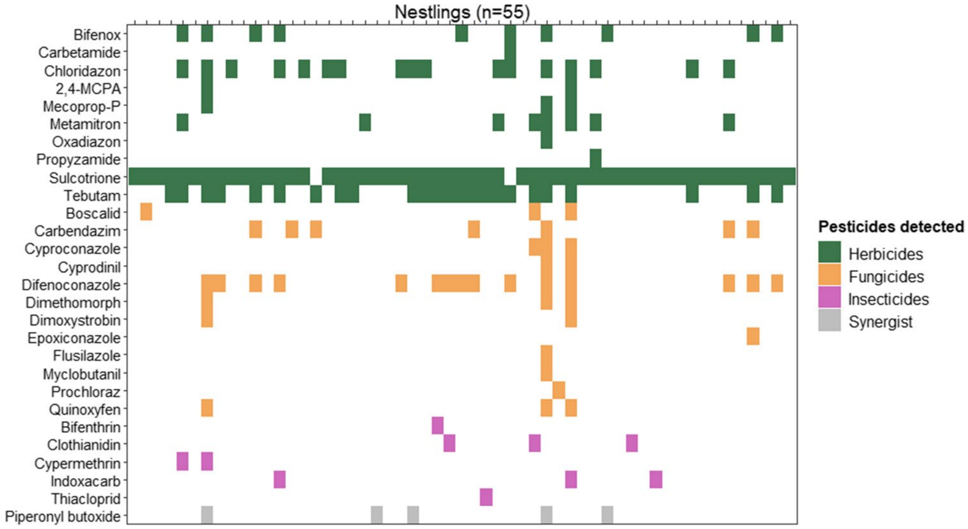

Fig. 2 Pesticide contamination patterns in *Circus pygargus* nestlings. Columns correspond to nestlings and rows correspond to compounds. Each coloured cell corresponds to a detected compound

Discussion

The present study revealed a general contamination to pesticides of Montagu's harrier chicks, and although only 27% of compounds searched for were detected, all chicks were contaminated. Herbicides and fungicides were most abundant in chicks (22 of 28 compounds), with three herbicides (sulcotrione, tebutam and chloridazon) detected at the highest occurrences, and difenoconazole was the most abundant fungicide. Most substances found in chicks were considered non-toxic based on acute toxicity hazard classification. Following this classification, thiacloprid had the highest level of acute toxicity, although the concentration measured in chick's blood did not exceed 0.25% of the substance's LD50. Nonetheless, sulcotrione, the most prevalent substance detected, is classified in category 4 for acute toxicity and is considered carcinogenic, mutagenic and reprotoxic, which raises questions about the consequences of this contamination for chicks' health.

Previous studies reported the contamination of wild birds with boscalid, cypermethrin, difenoconazole, indoxacarb, oxadiazon, sulcotrione, thiacloprid and piperonyl butoxide as detected here (Millot et al. 2015; Varagiya et al. 2021; Rial-Berriel et al. 2021; Fernández-Vizcaíno et al. 2022; Movalli et al. 2023). However, these studies used different biological matrices (organs such as gizzards or livers); thus, the comparison with our results is senseless given the different temporal patterns of pesticide distribution in the different biological tissues (Espín et al. 2016). Only a limited number of studies screened the same compounds we detected here in blood of wild birds. In Montagu's harriers from Germany, 2,4-MCPA was also detected in blood of two nestlings at 1.3 and 1.8 pg µL^{-1} (which is equivalent to pg mg^{-1} of blood considering a density of ~ 1) which is a thousand times lower than the minimal concentration found here (Badry et al. 2022). Twenty-one of the 28 compounds detected here were also found in fledglings of the European Kingfisher (*Alcedo atthis*) sampled the same year in western France (Musseau et al. 2023). Sulcotrione was notably found at high occurrence as well (95% of sampled birds) and at similar average concentrations (1277 pg mg^{-1} in Musseau et al. 2023*vs* 1112 pg mg^{-1} here). For 12 compounds, the average concentrations found in Montagu's harrier nestlings were higher than those found in the European Kingfisher. Cyproconazole, difenoconazole and flusilazole have been found in plasma of blackbirds (*Turdus merula*) with maximal concentrations of 0.212, 0.157 and 0.016 pg mg^{-1}, respectively (Angelier et al. 2023) *vs* 148, 1214 and 138 pg mg^{-1} in Montagu's harrier blood. Clothianidin

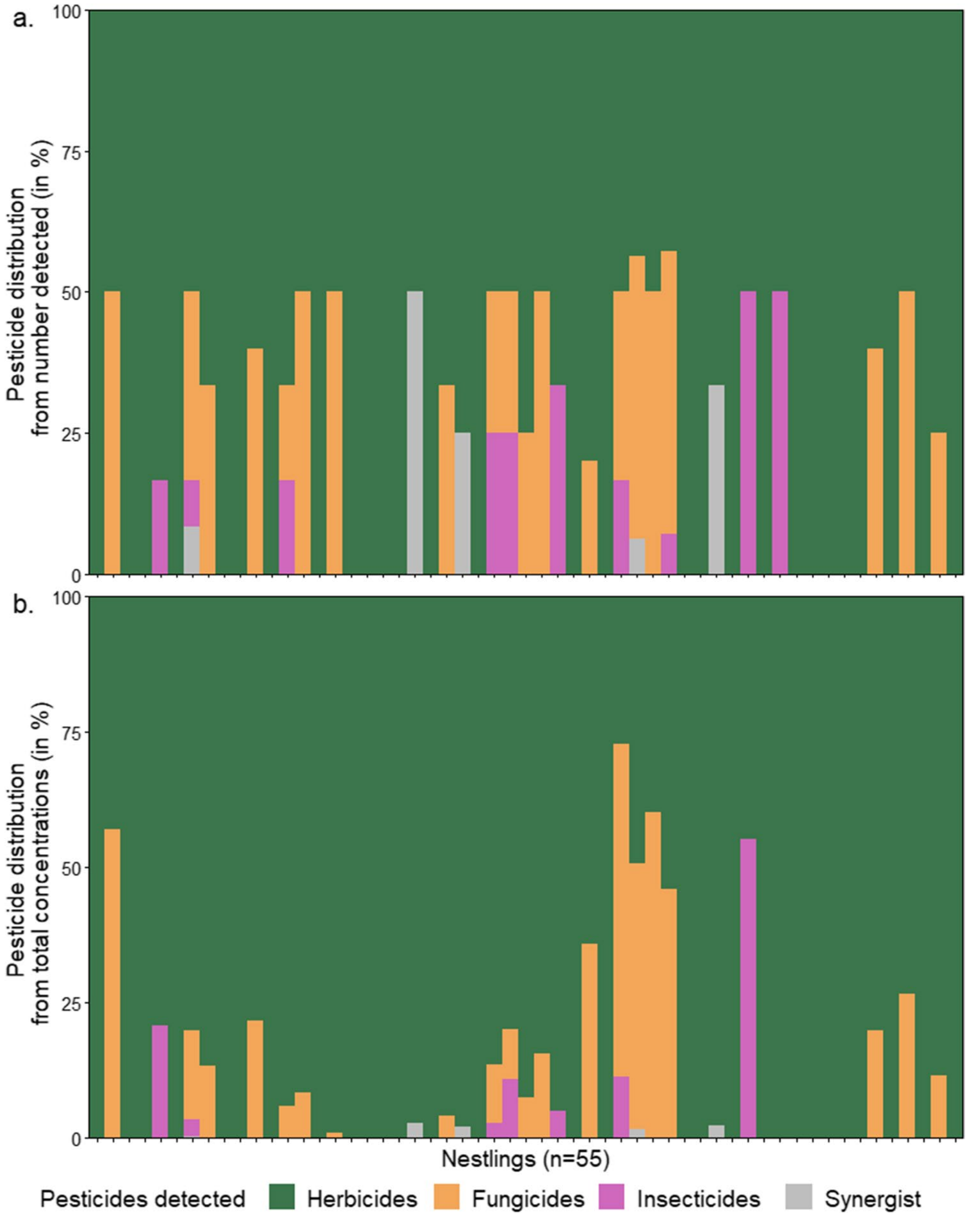

Fig. 3 Distribution of pesticide classes in blood samples of *Circus pygargus* nestlings according to their **a** number detected and **b** concentrations in pg mg^{-1}. One stacked bar corresponds to one nestling. When compounds were detected but not quantified, concentrations were estimated to be equal to the LOQ divided by 2

and thiacloprid have also been found at lower concentrations in blood of birds of different trophic levels within our study area and elsewhere (Rial-Berriel et al. 2020; Fuentes et al. 2023 and references therein). Differences in the sensitivity of the analytical methods used in the mentioned studies influence the occurrence and concentrations reported; comparisons should thus be considered with caution (except Fuentes et al. 2023; Musseau et al. 2023 and the present study).

Contamination of Montagu's harrier chicks was mostly herbicides and fungicides, indicating heavy use of these classes of pesticides in the study area and/or higher exposure of chicks to these classes due to specific ecological factors of this raptor species. Indeed, Montagu's harriers nest on the ground in cereal crops, which are dominant in the study area and mainly treated with fungicides and herbicides in France (DRAAF 2017). The large quantities of herbicides and fungicides bought into the study area support their heavy use, despite some mismatches between the amounts purchased and detection in nestlings; for example, propyzamide was bought in large amounts around nests but detected in only one nestling (Table 2 and Figure S1 in Supplementary Information). In fact, the quantities of substances bought and applied may vary according to the concentrations of active ingredients in PPPs, the application guidelines for PPPs (quantity to apply per hectare) and the proportions of different crop types surrounding nests. For instance, a higher proportion of corn crops locally may

Table 2 Compounds detected in the blood of 55 *Circus pygargus* chicks belonging to 22 nests, ordered by occurrence (highest to lowest). Means, standard deviation (SD) and minimum and maximal values were obtained from concentrations quantified in pg mg^{-1} in chicks' blood (i.e., values < LOD were excluded from calculations)

Compound	Detection (number of chicks)	Percentage	Mean ± SD	Min–max
Sulcotrione	53	96.36	1111.78 ± 540.67	312.42–3184.67
Tebutam	24	43.64	64.62 ± 49.77	13.81–180.24
Chloridazon	17	30.91	121.02 ± 125.90	35.96–563.10
Difenoconazole	15	27.27	240.02 ± 317.74	31.68–1213.55
Bifenox	10	18.18	< LOQ	-
Metamitron	8	14.55	25.01 ± 28.29	3.28–88.34
Carbendazim	7	12.73	97.50 ± 81.21	0.216–258.29
Piperonyl butoxide	5	9.09	36.30 ± 22.48	12.60–70.67
Boscalid	3	5.45	1369.09 ± 819.48	791.50–2307.00
Clothianidin	3	5.45	929.30 ± 1268.76	189.92–2394.32
Cyproconazole	3	5.45	70.04 ± 69.82	13.65–148.13
Dimethomorph	3	5.45	241.49 ± 94.27	163.33–346.19
Indoxacarb	3	5.45	< LOQ	-
Mecoprop-P	3	5.45	799.35 ± 369.40	409.93–1144.79
Quinoxyfen	3	5.45	39.93 ± 38.73	9.73–83.59
Cypermethrin	2	3.64	204.34 ± 46.88	171.19–237.49
Cyprodinil	2	3.64	41.34 ± 42.91	11.00–71.68
Dimoxystrobin	2	3.64	171.79 ± 16.87	159.86–183.72
2,4-MCPA	2	3.64	2020.12 ± 176.57	1895.27–2144.98
Bifenthrin	1	1.82	18.46	-
Carbetamide	1	1.82	29.75	-
Epoxiconazole	1	1.82	51.05	-
Flusilazole	1	1.82	137.63	-
Myclobutanil	1	1.82	142.12	-
Oxadiazon	1	1.82	71.68	-
Prochloraz	1	1.82	1292.89	-
Propyzamide	1	1.82	339.07	-
Thiacloprid	1	1.82	87.48	-

result in higher application of sulcotrione, whereas more beet crops might lead to greater use of metamitron, irrespective of the amounts bought at a larger scale. Nonetheless, the general pattern of pesticide use in the study area is reflected in the contamination of Montagu's harrier chicks. Furthermore, some compounds appear to be ubiquitous in the agroecosystem; boscalid, cyproconazole, prochloraz and thiacloprid have also been detected in soils, earthworms and small mammals in the study area (Pelosi et al. 2021; Fritsch et al. 2022).

The higher exposure of chicks to herbicides and fungicides implies the persistence of these substances for several weeks or even months in crop plots. Indeed, application of PPPs to cereal crops generally takes place in winter but can be extended until May for fungicides, coinciding with the onset of the breeding period for Montagu's harriers. This may expose chicks on the ground to persistent compounds through contact with the soil and vegetation, or through ingestion of contaminated prey. The concomitant detection of 16 compounds including difenoconazole, metamitron and carbendazim in small mammals sampled in the study area (Fritsch et al. 2022) supports a dietary contamination route. Higher concentrations were found in raptor chicks than in small mammals (843-fold higher on average; Table S3), which suggests a potential biomagnification of these compounds up the trophic chain. For recently banned compounds, their presence in the blood of Montagu's harrier chicks can be rationally explained by the delay afforded to distributors and users of PPPs. For example, chloridazon, a substance banned in France in 2018 but detected in 30% of Montagu's harrier nestlings and in small mammals (Fritsch et al. 2022), was bought into the study area in 2020 (Figure S1 in Supplementary Information). This compound was purchased as a PPP mixture with quinmerac with an end date for distribution of June 2020 and an end date for use of December 2020. Thus, the application and persistence of this compound until chicks were raised during the summer of 2021 may be the origin of their contamination. However, regarding compounds that have been banned for a long time, their detection implies either fraudulent use or strong persistence in the environment. The persistence of a compound is generally established from its 50% detection time (DT50), the time taken to detect a 50% decrease in pesticide concentration under controlled conditions in either laboratory or field. For tebutam, the DT50 is 60 days in the field, meaning that this molecule is supposed to be naturally degraded within 2 months in the environment (Lewis et al. 2016). Based on our results, its rate of degradation would be much slower than predicted, which can be explained by the gap between *in natura* conditions and the conditions to establish the DT50 (Moreau et al. 2022), and plants would remobilise this contaminant from the soil 20 years after its ban, which seems quite unlikely. However, the detection of the banned compounds tebutam, chloridazon, epoxiconazole, quinoxyfen, bifenthrin and clothianidin in the blood of European Kingfisher's fledglings sampled the same year at ~ 100 km of our study area raises questions concerning the potential fraudulent use of such pesticides (Musseau et al. 2023).

If we discount the fraudulent use of legacy substances, their presence in Montagu's harrier chicks raises questions about the aforementioned exposure pathways (i.e., contact and diet). Thus, another contamination route might be the maternal transfer of pesticides. Indeed, if these substances are currently used in western African countries, where this raptor species overwinters, females may be exposed before arriving to their breeding site, then detoxify themselves through egg-laying. Maternal transfer of pollutants is a well-known process for persistent molecules and heavy metals (Mineau 1982; Van den Steen et al. 2009; Jouanneau et al. 2021). More recently, some studies demonstrated the maternal transfer of 'non-persistent' pesticides such as tebuconazole (Bellot et al. 2022). Lipophilic molecules are generally more prone to be excreted by females in the vitellus of their eggs (Fry, 1995). Flusilazole, a triazole fungicide just as tebuconazole, and tebutam have high and moderate lipophilicity, respectively (see log P values in Table 1), suggesting this contamination pathway should not be excluded. Further investigations on pesticide use in African countries and in migratory stopover areas are needed to assess maternal transfer of these pesticides in Montagu's harriers.

Regardless of the route of exposure, our study provides evidence that 'naïve' individuals such as Montagu's harrier chicks are contaminated with pesticide mixtures after only 4 weeks of life within crop plots. This highlights the ubiquity of pesticides in agroecosystems, including some that have been banned for many years. Although most studies consider the greater risk of adverse effects of insecticides on wildlife, our study also highlights, in line with previous studies, the need to consider herbicide and fungicide risks to non-target organisms into more details as these were the most prevalent compounds found here (Tassin de Montaigu and Goulson 2020). Besides, even if not discussed in the present study, there seems to be a quite large variability in contamination among nestlings, and further investigations are needed to determine if this could have implications for the use of Montagu's harrier chicks in biomonitoring schemes. Ongoing research on soils and earthworms in the study area should help to disentangle the origin and exposure routes for these contaminants. Moreover, dietary exposure could be investigated by analysing pesticides in food pellets collected at nests. Blood sampling of breeding adults and of younger nestlings would also be of great interest for studying the potential maternal transfer of pesticides *in natura*. Additionally, given the mixtures (16 compounds in one nestling) and the toxicity of some of the substances detected, further investigations are needed to shed light on the effects of pesticides on the life-history traits of chicks and adults. This would help to determine the consequences of pesticide exposure on the health of Montagu's harriers and eventually humans in a *One Health* framework.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by EF, AR, MM and KM. The first draft of the manuscript was written by EF. EF, AR and KM performed the writing. JM, VB and KM supervised, commented and edited on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The data that support the findings of this study are not openly available but are, however, available from the authors upon reasonable request.

Declarations

Ethical approval This study was conducted following the French guidelines for the ethical use of animals in research (APAFIS#18557–2019010822312199v2). Handling of Montagu's harriers was licensed by the Centre de Recherches sur la Biologie des Populations d'Oiseaux – Museum National d'Histoire Naturelle (license #1308).

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Supplementary Information

Pesticide contamination patterns in Montagu's harrier (*Circus pygargus*) chicks

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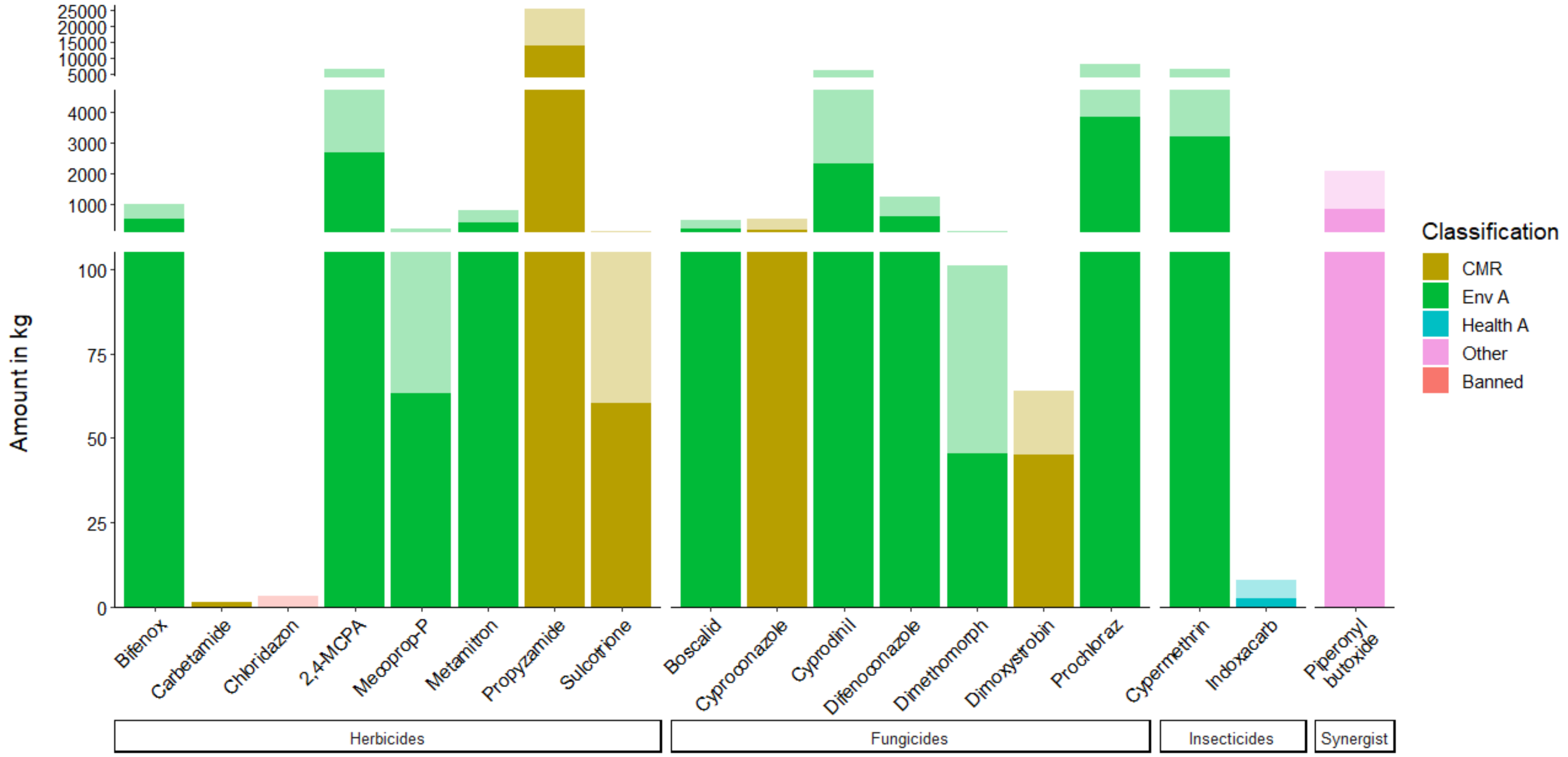

Figure S1. Quantities of substances bought in 2020 (faded stacked bars) and 2021 (coloured stacked bars) in areas surrounding nests. Quantities of substances bought in kg were summed from postal codes within a 14 km radius around nests, which corresponds to the average foraging range for Montagu's harriers in the study area (Salamolard 1997) and were compiled from a database of the national sales bank of distributors of plant protection products (BNVD, 2023; see Table S1). Substances were classified according to their danger level following the nomenclature of legislation on substances relating to the fee for diffuse pollution (Order of 22nd December 2022; Table S2). Banned = substances banned for sale and use in France before 2021. Ten of the substances detected are not included in the graphic because they were banned and/or not sold in 2020 or 2021.

Table S1. Criteria for classification of substances in acute and environmental/health toxicity hazards categories based on EC Regulation No. 1272/2008 and Order of 22nd December 2022.

Type	Classification	Criteria
Acute toxicity	1	LD50 ≤5 $mg.kg^{-1}$
	2	5 <LD50 ≤50 $mg.kg^{-1}$
	3	5 <LD50 ≤300 $mg.kg^{-1}$
	4	300 <LD50 ≤2000 $mg.kg^{-1}$
	-	LD50 >2000 $mg.kg^{-1}$
Environmental/ Health toxicity	CMR	Carcinogenic, mutagenic in germ cells, or toxic for reproduction
	Health A	Acute toxicity of category 1, 2 or 3, or specific toxicity toward target organs of category 1 after single or repeated exposure, or due to their effects on breast-feeding.
	Env A	Acute toxicity to aquatic environments of category 1 or chronic toxicity to aquatic environments of category 1 or 2.
	Other	Other substances

Table S2. Classification in acute and environmental/health toxicity hazard categories for the 28 substances detected in Montagu's harrier chicks. Categories of toxicity are explained in Table S2. Substances banned before 2021 are in bold italics.

Substances	Acute toxicity hazard	Environmental/Health toxicity hazard
Herbicides		
Bifenox	-	Env A
Carbetamide	-	CMR
Chloridazon	**-**	**-**
2,4-MCPA	4	Env A
Mecoprop-P	4	Env A
Metamitron	4	Env A
Oxadiazon	**-**	**-**
Propyzamide	-	CMR
Sulcotrione	4	CMR
Tebutam	**-**	**-**
Fungicides		
Boscalid	-	Env A
Carbendazim	**-**	**-**
Cyproconazole	3	CMR
Cyprodinil	4	Env A
Difenoconazole	-	Env A
Dimethomorph	-	Env A
Dimoxystrobin	-	CMR
Epoxiconazole	**-**	**-**
Flusilazole	***4***	**-**
Myclobutanil	4	x
Prochloraz	4	Env A
Quinoxyfen	**-**	**-**
Insecticides		
Bifenthrin	***4***	**-**
Clothianidin	***4***	**-**
Cypermethrin	-	Env A
Indoxacarb	3	Health A
Thiacloprid	***2***	**-**
Synergist		
Piperonyl butoxide	-	Other

Table S3. Compounds detected in fur of small mammals (Fritsch et al. 2022) and blood of Montagu's harrier chicks (this study) in the study area. Maximum concentrations are given in pg.mg^{-1}. The increase factor indicates the number of times small mammal concentrations are multiplied to reach chick concentrations.

Compounds	Small mammals	Montagu's harrier chicks	Increase factor
2,4-MCPA	428.000	2144.98	5
Boscalid	355.000	2307.00	6
Carbendazim	200.000	258.29	1
Chloridazon	0.766	563.10	735
Clothianidin	1.140	2394.32	2100
Cypermethrin	1.340	237.49	177
Cyproconazole	77.800	148.13	2
Cyprodinil	14.600	71.68	5
Difenoconazole	0.157	1213.55	7730
Flusilazole	0.107	137.63	1286
Mecoprop-P	133.000	1144.79	9
Metamitron	5.240	88.34	17
Oxadiazon	0.054	71.68	1327
Prochloraz	22.000	1292.89	59
Propyzamide	270.000	339.07	1
Thiacloprid	2.670	87.48	33

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Order of 22nd December 2022 establishing the list of substances defined in article L. 213-10-8 of the Environment Code relating to the fee for diffuse pollution. *Journal officiel électronique authentifié n° 0301 du 29/12/2022*. https://www.legifrance.gouv.fr/eli/arrete/2022/12/22/TREL2235282A/jo/texte. Accessed 16 November 2023 (in French).

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Salamolard, M. (1997). Utilisation de l’espace par le Busard Cendré *Circus pygargus*, superficie et distribution des zones de chasse. Alauda, 65, 307–320 (in French).

ARTICLE 3

ORGANIC FARMING REDUCES PESTICIDE LOAD IN A BIRD OF PREY

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Organic farming reduces pesticide load in a bird of prey

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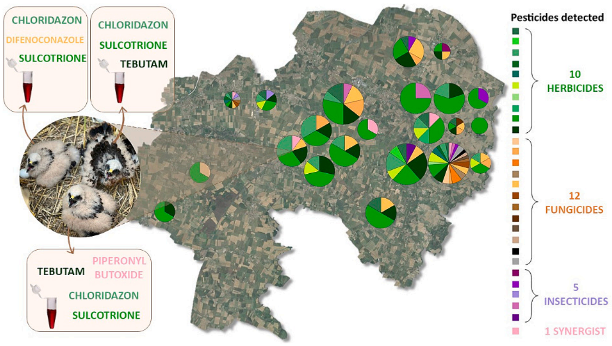
HIGHLIGHTS

- No study exists on inter- and intra-brood variability of non-persistent pesticides.
- 28 compounds were detected in blood of 55 *Circus pygargus* chicks from 22 nests.
- There is a high variability of contamination levels within and among nests.
- More organic farming around nests reduces the number of pesticides in chicks' blood.
- Neither sex nor its interaction with hatching order influences chicks' contamination.

GRAPHICAL ABSTRACT



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ABSTRACT

Human activities have led to the contamination of all environmental compartments worldwide, including bird species. In birds, both the environment and maternal transfer lead to high inter-brood variability in contamination levels of pollutants, whereas intra-brood variability is generally low. However, most existing studies focused on heavy metals or persistent compounds and none, to our knowledge, addressed the variability in contamination levels of multiple pesticides and the factors influencing it. In this study, the number of pesticides detected (of 104 compounds searched) and the sum of their concentrations in the blood of 55 Montagu's harrier (*Circus pygargus*) nestlings from 22 nests sampled in 2021 were used as metrics of contamination levels. We investigated the effect of organic farming at the size of male's home range (i.e., 14 km^2) and chicks' sex and hatching order on contamination levels. We did not find a difference between inter-brood and intra-brood variability in pesticide contamination levels, suggesting a different exposure of siblings through food items. While chicks' sex or rank did not affect their contamination level, we found that the percentage of organic farming around the nests significantly decreased the number of pesticides detected, although it did not decrease the total concentrations. This finding highlights the potential role of organic farming in reducing the exposure of birds to a pesticide cocktail.

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1. Introduction

Anthropogenic pollution associated with industrialisation, urbanization, and agricultural intensification has led to the contamination of multiple environmental compartments (i.e., biotic and abiotic elements), notably wildlife (Mañosa et al., 2001; Perugini et al., 2011). For decades, studies have investigated the factors influencing the exposure of marine bird species to contaminants *in natura*, mostly heavy metals and Persistent Organic Pollutants (POPs) including perfluoroalkyl and polyfluoroalkyl substances and organochlorine pesticides such as DDT (Becker, 1992; Hario et al., 2000; Janssens et al., 2002; Custer et al., 2010; Jouanneau et al., 2021). Various contamination pathways for these pollutants have been identified; contamination may occur through direct contact with contaminated environments, ingestion, and maternal transfer (Becker and Sperveslage, 1989; Jouanneau et al., 2021). Direct contact or ingestion of contaminated food is supposed to be reduced with the increasing distance to the pollution source. For instance, levels of lead in feathers of house sparrows showed an urbanization gradient, that is, higher levels of contamination closer to heavily urbanized areas (Bichet et al., 2013). Moreover, geographical factors influence the spatial and temporal trends in contamination levels (Devalloir et al., 2023). In addition, the food brought by adults to the nest may show variation in contaminant burden; thus, variation in prey selection and foraging habitat preferences of parents may explain the differences in chick contamination between broods (Goutner et al., 2001). Thus, in general for heavy metals and POPs, inter-brood variability in contamination patterns is attributable to both direct environmental factors associated with the nest location and food provisioning by adults.

In species such as the peregrine falcon (*Falco peregrinus*; Newton et al., 1989) and common tern (*Sterna hirundo*; Power et al., 2021), eggs from the same clutch have been reported to show similar contamination levels; however, some other species show great variation within a clutch according to the egg laying sequence (e.g., American avocets *Recurvirostra americana*, black-necked stilts *Himantopus mexicanus*, and Forster's terns *Sterna forsteri*; Ackerman et al., 2016). In general, maternal transfer of contaminants increases with the laying order, with the last laid egg being the most contaminated, because egg laying may be used as a detoxification mechanism in females (for instance in herring gulls *Larus argentatus*; Mineau, 1982; Robinson et al., 2012). However, some studies have reported that the first laid egg is the most contaminated one, indicating a reversed detoxification pattern of females (in herring gulls and common terns; Becker et al., 1994; blue tits *Cyanistes caeruleus*; Van den Steen et al., 2009; Audouin's gull *Larus audouinii*; Vicente et al., 2015). However, these findings vary not only with species but also with the type of contaminant studied (Janssens et al., 2002; see Table 1 in Van den Steen et al., 2009). Similarly to eggs, hatchlings display contamination patterns that reflect the contamination levels of the breeding female and the detoxification pattern in egg laying sequence, that is, the highest contamination levels may be observed in either the first-hatched or the last-hatched offspring (Lemmetyinen et al., 1982; Becker and Sperveslage, 1989; Becker et al., 1993). Nevertheless, the contamination pattern of chicks also depends on food (Becker and Sperveslage, 1989), and one may expect siblings to display the same contamination levels, as they share the same environment and food. However, access to prey items may vary within the same clutch owing to variations in the morphological, physiological, and behavioural traits of chicks and the feeding strategy of parents (Viñuela, 1999), as hatching asynchrony generally leads to competitive and developmental hierarchies among chicks (Love et al., 2003). For instance, in the brown falcon, *Falco berigora*, the last-hatched chicks displayed lower growth rates and body condition and a higher probability of mortality than chicks hatched earlier (McDonald et al., 2005). Nonetheless, these differences are not observed in all species exhibiting hatching asynchrony (Krebs, 1999). Consequently, it is difficult to predict the direction of eggs and nestlings' contamination levels based on their laying and hatching order, respectively. Intra-brood variability may also occur because of sex-related differences among chicks. This is especially the case in dimorphic bird species, in which one of the sexes exhibits larger body size. The larger and thus faster-growing chicks may benefit from a "dilution effect" as the higher tissue growth rate is greater than the rate of contaminant deposition in them (Stewart et al., 1997; Goutner et al., 2001; Ackerman et al., 2011). Moreover, food provisioning leads to sibling competition, with usually the larger sex being favoured (Anderson et al., 1993). In Montagu's harriers, *Circus pygargus*, first-laid eggs are more likely to produce females, the larger sex, which are produced in higher numbers than males especially in years of high food abundance (Leroux and Bretagnolle, 1996; Arroyo, 2002). Consequently, sex and hatching order are key characteristics leading to dominant–subordinate hierarchies among nestlings during feeding events, as larger individuals might outcompete the smaller ones and gain better access to prey items (Anderson et al., 1993). Thus, intra-brood variability in contamination patterns may be attributable to both rank and sex of the chicks, which set their initial contamination level and affect their ingestion of contaminants.

Although studies have examined the inter- and intra-brood variability in contamination levels in birds for decades, few have focused on non-persistent pesticide contaminants (i.e., excluding POPs), and none—to our knowledge—have investigated multiple pesticides other than chlorinated pesticides (Custer et al., 2010; Bustnes et al., 2015). A first reason was likely the absence of technical means to simultaneously detect and quantify multiple molecules. However, suitable analytical methods are now available (Rodrigues et al., 2023); therefore, pesticide contaminants should be investigated. Agricultural intensification has led to the extensive use of pesticides and widespread contamination of agroecosystems; consequently, synthetic fertilizers and pesticides are found in both abiotic and biotic environmental compartments (Székács et al., 2015; Wintermantel et al., 2020; Fritsch et al., 2022; Fuentes et al., 2023a). Organic farming is a production system that excludes the use of synthetic farm inputs (Lockeretz, 2007). Therefore, the application of organic farming practices is expected to reduce contamination levels in the environment and consequently in wildlife. In an analysis of topsoil samples collected across Europe, samples from organic farms showed significantly fewer pesticide residues and in lower concentrations than those from conventional farms (16 vs. 5 residues/sample and 70 to 90 % lower concentrations; Geissen et al., 2021). Pesticide mixtures have also been reported to be lower in both soils and earthworms sampled in organic farms than in those from conventional farms (Pelosi et al., 2021); however, some studies report contradictory findings, with no effect of the practices on the contamination levels in small mammals (Fritsch et al., 2022). This may result from unforeseen contamination of organic fields from either persistence of molecules or large-scale drifts of pesticide particles during spraying or as dust or runoff waters (Humann-Guilleminot et al., 2019; Wintermantel et al., 2020). Still, a larger proportion of organic farms (i.e., lower proportion of conventional farms applying pesticides) in the landscape should reduce the unintentional pesticide contamination of untreated fields and thus the global exposure of wildlife to pesticides.

The Montagu's harrier is a declining bird of prey species, specialist in agroecosystems, nesting on the ground, generally of cereal fields. Consequently, their eggs and chicks are directly exposed to local pesticide contamination throughout their growth period, through direct spraying on eggs, contact with contaminants remaining on the soil and on the crop, and through feeding on contaminated prey. Montagu's harriers mainly prey on voles and feed upon orthopterans and passerine birds as alternative prey (Salamolard et al., 2000). Males are the main sex involved in providing food resources, hunting at large distances from the nest (5.8 ± 4.1 km; Guixé and Arroyo, 2011); females also provide food from the surroundings (<1 km), mainly before fledging, especially in years of low food abundance (Garcia and Arroyo, 2005; Wieringa et al., 2019). The species is sexually dimorphic; females are ~19 % heavier than males, and this difference becomes detectable during the second part of the rearing period (Millon and Bretagnolle, 2005), which may lead to sibling competition for food. Nevertheless, because chicks of the same brood share the same parents, environment, and food, they are expected to show similar contamination patterns those eventually differ from the ones observed in chicks from another brood.

The present study aimed at investigating the inter- and intra-brood variability in pesticide contamination found in Montagu's harrier chicks and the factors influencing this variability. We used the number of pesticides detected and the total sum of concentrations of pesticides in chick blood as proxies of contamination levels. Indeed, these metrics have been commonly used in ecotoxicology studies to describe individual or environmental contamination loads (Tartu et al., 2014; Fritsch et al., 2022; Zaller et al., 2022). Assuming an additive effect of pesticides, a higher number of compounds and/or higher total concentrations are expected to increase the number of potential interactions among mixture components and thus the risk of cocktail effects (Hernández et al., 2017; Zaller et al., 2022). We first investigated the relative inter-brood vs. intra-brood variability in contamination levels in chicks. Then, we tested the effects of the proportion of organic farming at the scale of male's home range (i.e., 14 km^2, Salamolard, 1997, see below for details). Lastly, we tested the effects of sex and hatching order of chicks on contamination levels. We expected to find a higher inter-brood variability than intra-brood variability and lower contamination levels with a higher proportion of organic farming. We also expected to identify a relationship between the sex and hatching rank of chicks and the contamination levels, with females of rank 1 being less contaminated because of a "dilution effect" and a higher pesticide load in last-laid eggs.

2. Materials and methods

2.1. Study site and model species

The study site is a 450 km^2 area located in southwestern France (46°11′N, 0°28′W) in the Long-Term Social-Ecological Research platform, Zone Atelier Plaine & Val de Sèvre (LTSER ZAPVS). Every year, crop identification is performed in this area, allowing mapping of organic crop plots in detail (Bretagnolle et al., 2018, see also Fuentes et al., 2023b; Fig. 1). In France, organic farming complies with the European legislation on organic production, and thus synthetic pesticides and fertilizers to grow crops are banned. In the ZAPVS, Montagu's harrier nests have been monitored since 1994 (Bretagnolle et al., 2018). These birds nest on the ground in cereal crops and lay up to eight eggs (Arroyo et al., 1998). Eggs are incubated by females for 29 days, and chicks are reared in the nest for 30–35 days (Arroyo et al., 2007). At this site, the mean productivity is reported to be 2.05 fledglings per breeding attempt (Arroyo et al., 2004), breeding success depending mainly on the availability of its main prey, the common vole (*Microtus arvalis*; Salamolard et al., 2000). In years of poor vole availability, Montagu's harriers also feed on orthopterans (Butet and Leroux, 2001). Nestling food is

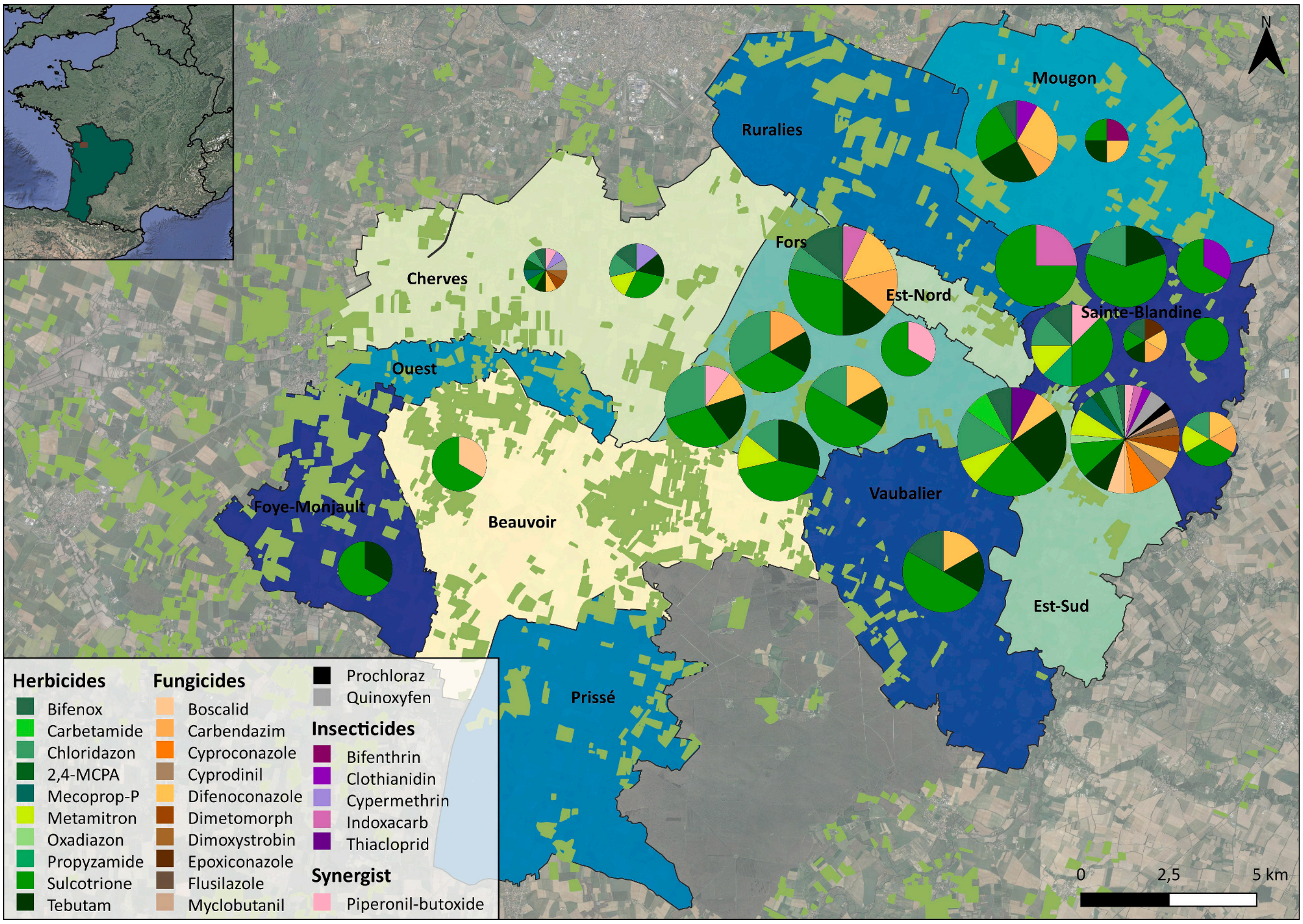

Fig. 1. Pesticide contamination of Montagu's harrier chicks from the Zone Atelier Plaine & Val de Sèvre (ZAPVS) in 2021. For each nest, a pie chart represents the proportion of each molecule detected in the chicks from the corresponding nest. Pesticide molecules are classified according to their target group: insecticides, herbicides, fungicides and synergist. The size of the pie chart reflects the brood size—that is, the larger the size of the pie chart, the higher the number of chicks were sampled—and its localisation corresponds to the approximate nest localisation across the sectors of the study area. In total, 22 nests corresponding to 55 nestlings are represented. Organic crop plots in 2021 are depicted in light green.

mainly provided by males, and females contribute more towards the end of the rearing period, usually by hunting close to the nest (Garcia and Arroyo, 2005). During the breeding season, home ranges and hunting distances vary according not only to sex and breeding phase (incubation, nestling, fledgling), but also to the study area and the year's food abundance (Wieringa et al., 2019; Krupiński et al., 2020; Berger-Geiger et al., 2022). In the study area, the home range of males is ~14 km^2 (Salamolard, 1997). To cover their home range, we considered zones within a radius of 2200 m around nests as foraging areas thus corresponding to an area of ~15 km^2.

2.2. Sampling procedure

Between May 2021 and early August 2021, professional ornithologists located and visited all nests in the study area, which were then mapped using global positioning system (GPS) coordinates. Nest coordinate data were stored on a geographical information system (GIS; QUANTUMGIS 3.16.5; QGIS Development Team, 2023; Fig. 1). The nests were visited twice before eggs hatched and every week subsequently (lag time of 7 ± 2 days between visits). During the hatching visit, chicks were head-marked (using water-based coloured pens) allowing their individual identification. Once chicks were 15 days old, they were sexed according to the colour of their iris (brown for females and grey for males; Leroux and Bretagnolle, 1996) and banded with a unique coded aluminium ring provided by the Museum National d'Histoire Naturelle de Paris (France). When nestlings were 26 ± 2 days old, they were caught, carefully handled in the shortest time possible to collect blood samples and released at their nest. For each chick, 50 μL of blood was collected by puncturing the brachial vein using a sterile needle and heparinised capillary tubes. Samples were placed in plastic microcentrifuge tubes and stored and transported in a cooler (0–5 °C) until they were brought to the laboratory, where they were stored at −20 °C for further analyses.

In total, 70 chicks from 24 nests hatched during the study period, of which 12 died before fledging and 3 fledged but were not sampled, representing a total of 55 chicks from 22 nests sampled. Of the 22 nests studied, 13 had >2 fledglings sampled, and 4 nests had up to 4 fledglings at the last visit. The ranking procedure that consisted in attributing the rank 1 to the oldest chick (i.e., the first-hatched) accounted thus for missing siblings that died before the last visit in these broods (7 chicks), using data from previous visits so that the ranking matched the hatching order. For 85 % of chicks, hatching order was determined during the hatching visit through head-marking of chicks. For the remaining 15 % of chicks, *a posteriori* determination of age was made using a morphometric estimation of age at last visit (function using wing length measure [Arroyo, 1995]; calculator available at https://busards.com/index.php/Outils/biometry). In 2021, the time between two hatching events within broods was on average 1.69 days (± standard deviation: 0.94).

No sex ratio bias was observed among the 55 chicks sampled during the study period (29 females and 26 males; binomial test $p = 0.53$) or among each rank class (binomial test at rank 1 $p = 0.50$ ($n = 20$), rank 2 $p = 0.23$ ($n = 18$), rank 3 $p = 0.06$ ($n = 14$) and rank 4 $p = 1$ ($n = 3$)).

2.3. Pesticide analysis

Pesticide extraction was conducted following the method developed by Rodrigues et al. (2023). In brief, blood samples were concentrated and subjected to multiresidue analysis using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) and automated thermal desorption gas chromatography coupled to tandem mass spectrometry (ATD-GC–MS/MS) (see Rodrigues et al., 2023 for the complete methodology). Inter and intra-day variability in precision is taken into account through the injection of a standard at the start of each series of analyses, to check if the analytical equipment is giving the expected results. A total of 104 compounds were searched, including herbicides, fungicides, insecticides, a synergist and a safener (see Rodrigues et al., 2023 for the complete list of compounds searched). The method used for each compound detected and limits of detection (LOD) and quantification (LOQ) values are provided in Supplementary material (Table S1).

2.4. Statistical analyses

The contamination levels of chicks were determined using two metrics, the number of pesticides detected and the sum of the concentrations of pesticides in chick's blood. All analyses were performed using R v.4.2.2 software (R Core Team, 2022). Both response variables (number of pesticides and sum of concentrations) data did not meet the normality and homoscedasticity assumptions; therefore, before model implementation, sum of concentrations were log-transformed and the normal distribution fit was visually checked using the fitdist function from the "fitdistrplus" package (Delignette-Muller and Dutang, 2015). For the number of pesticides variable, the distribution with the best fit—Poisson or negative binomial distribution—was selected using the goodness-of-fit criteria from the gofstat function of the same package. The "lme4" package was used to run the linear mixed-effects model (LMM) for the sum of concentrations (log-transformed) and the generalized LMM fitted with a negative binomial distribution (NBGLMM) for the number of pesticides (Bates et al., 2015). For each model detailed hereafter, residuals were checked using the "DHARMa" package (Hartig, 2022) and compared using likelihood ratio-based χ^2-statistics.

To investigate whether the intra-brood variability in contamination levels was lower than the inter-brood variability (i.e., whether chicks from the same nests had similar contamination levels in comparison with chicks from different nests), we assessed repeatability using the intraclass correlation (ICC) coefficient, which is the ratio of between-groups variance to total variance (the sum of between-groups and within-groups variance) (Nakagawa and Schielzeth, 2010; Nakagawa et al., 2017; Carrasco, 2022a), the group being the nest identity in our case. Thus, the value of this metric lies between 0 and 1: if it equals 1, the total variance is attributable to inter-brood variability, and if it is 0, the total variance is attributable to intra-brood variability. Contamination level repeatability of chicks within nests was obtained using the rpt function from the "rptR" package v.0.9.22 (Stoffel et al., 2017), with the log-transformed sum of concentrations as the outcome and the nest identity as grouping factor (the random factor of the LMM) and using the icc_counts function from the "iccCounts" package v.1.1.1 (Carrasco, 2022b), with the number of molecules as outcome, the nest identity as subjects (the random factor of the GLMM), and negative binomial distribution family.

To test the effect of organic farming in local foraging areas on the level of contamination of chicks, the log-transformed sum of concentrations was used as response variable in an LMM using the percentage of organic farming (log-transformed) as a fixed-effect and similarly for the number of pesticides detected, used as response variable in an NBGLMM. To avoid pseudo-replication, nest identity was included as a random effect (as chicks can belong to the same nest) (Bolker et al., 2009). As we wanted to investigate the effect of organic farming on the level of contamination of chicks at a local scale, we tested the percentage of organic farming around the nests for 20 buffer sizes corresponding to radii ranging from 100 to 2200 m, with an increment of 100 m, as described in Fuentes et al. (2023b). Thus, we ran 20 LMMs and 20 NBGLMMs (one per buffer size) and used the Akaike Information Criterion corrected for small sample size (AICc from "AICcmodavg" package, Mazerolle, 2023) to select the best model (i.e., having the lowest AICc), assuming that models with a difference in AICc <2 are similarly supported and cannot be distinguished from each other (Burnham and Anderson, 2004). The direction and significance of the effect of organic farming was determined using effect size (estimate) and its bootstrapped 95 % confidence interval (CI; see Fuentes et al., 2023b for further details). The factors influencing the intra-brood variability in contamination levels were assessed using the total pesticide concentrations (log-transformed) and the number of pesticides detected as the response variable in LMM and NBGLMM, respectively, and chick sex and hatching order and their interaction as fixed effects, including nest identity as a random effect.

3. Results

3.1. Pesticide load overview and variability

A total of 28 pesticides of the 104 searched were detected above the LOD in Montagu's harrier chicks: 10 herbicides, 12 fungicides, 5 insecticides and 1 synergist (see Supplementary material Table S2), among which 26 were quantified (i.e., above LOQ). The geographical distribution among the nests is depicted in Fig. 1. All chicks sampled ($n = 55$) were found to be contaminated with at least one pesticide, and the maximum number of pesticides detected per chick was 16 (mean ± standard deviation = 3.2 ± 3.1; Table 1). Total concentrations ranged from 24.41 to 7265.52 pg.mg^{-1} (1538.00 ± 1279.40 pg.mg^{-1}; Table 2). The relationship between the number of pesticides and the sum of concentrations is depicted in Fig. 2, the two metrics were not correlated to each other (Spearman's rank correlation test, $\rho > 0.21$, 95 % CI = [−0.07; 0.48], $n = 55$) even when considering females ($\rho > 0.26$, 95 % CI = [−0.19; 0.64], $n = 29$) and males ($\rho > 0.17$, 95 % CI = [−0.07; 0.48], $n = 26$) separately.

The estimated repeatability for the number of compounds (ICC mean ± standard error = 0.32 ± 0.16, 95 % CI = [−0.02; 0.60]) and the log-transformed sum of concentrations ($R = 0.08 \pm 0.12$, 95 % CI = [0; 0.40]) were low and not significant, indicating that there was no statistical difference of within and between nest variation (see Supplementary material Figs. S1 and S2).

3.2. Effects of organic farming

None of the buffer sizes was appropriate to analyse the effect of organic farming (log-transformed) on the total concentrations (log-transformed) as almost all models had a difference of AICc <2 (Fig. S3). The model with a buffer size of 300 m was best supported for the effect of the organic farming on the number of molecules, as it had the lowest AICc and no other model had an AICc difference < 2 (Fig. S4). In a zone with a radius of 300 m around the nests, a higher percentage of organic farming (log-transformed) significantly reduced the number of pesticides detected in chick's blood (effect size = −0.38; 95 % CI = [−0.66; −0.13]; Tables 3 and S3; Fig. 3). A higher proportion of organic farming around nests significantly decreased the number of pesticides in Montagu's harrier chicks both at the scale of the crop plot (300–500 m) and at a larger scale (1100–2200 m, see Fig. S4 in Supplementary material).

3.3. Effects of the sex and rank of chicks

The summary statistics of sex and rank factors are provided in Table 1 for the number of pesticides detected and in Table 2 for total pesticide concentrations. Rank 4 female chicks showed the highest number of pesticides detected with an average of 7.5 ± 9.2 compounds ($n = 2$), whereas rank 1 males showed the lowest mean with 2.5 ± 1.8 compounds ($n = 8$). Similarly for total pesticide concentrations, females of fourth rank had the highest average with 3876.52 ± 4792.75 pg.mg^{-1} and males of rank 2 the lowest with 896.58 ± 635.15 pg.mg^{-1} ($n = 6$). Although a tendency of increased contamination levels was observed for last-hatched chicks (Figs. 4b, 5b), there was no significant effect of rank neither on the number of pesticides detected nor on the log-transformed sum of concentrations (Table 3 and Tables S3, S4). Neither sex nor its interaction with the rank influenced the contamination levels of chicks (Table 3 and Tables S3, S4; Figs. 4 and 5).

4. Discussion

In a previous study, we investigated the potential of Montagu's harrier chicks for the biomonitoring of non-persistent pesticides, describing contamination patterns and the routes of exposure (Fuentes et al., 2024) while the present study aimed at characterizing the variation of pesticide contamination level of Montagu's harrier chicks among and within nests, and investigating the link with the composition of the surroundings in organic crops and with chicks' sex and rank. Thus, for further details concerning the description of compounds detected refer to Tables S1 and S2 in Supplementary material.

4.1. Pesticide load overview and variability

Pesticide loads in Montagu's harrier chicks were variable, some chicks being strongly contaminated (up to 16 pesticides and 7265.51 pg.mg^{-1}) and others having low contamination levels (1 compound and 24.41 pg.mg^{-1}). However, our finding concerning the intra-brood vs. inter-brood variability was unexpected as the intra-brood variability was similar to the inter-brood variability in pesticide load (Nakagawa and Schielzeth, 2010). In other words, chicks from the same nest did not differ in their contamination levels from chicks of other nests. This result contrasts with findings in the Squacco heron (*Ardeola ralloides*) where the variability in mercury levels in chicks' feathers was mostly attributable to inter-brood differences rather than intra-brood differences (Goutner et al., 2001). Subsequently, some studies suggested that sampling a random egg from a clutch would provide an appropriate estimation of the contamination of the whole clutch (Bustnes et al., 2015; Power et al., 2021). However, in the present study, it appears that sampling blood from a randomly chosen chick would have yielded an imprecise estimation of the nest's contamination. For instance, the number of compounds detected in blood samples from four siblings from the same nest was 6, 5, 2, and 1, and total concentrations were 2113.18, 426.06, 1081.94, and 1364.10 pg.mg^{-1}, respectively; thus, random sampling would have led to an overestimation or underestimation of the nest's contamination level. In that respect, caution should be exercised when choosing a sampling strategy (random or systematic) in

Table 1
Summary of the number of pesticides detected in Montagu's harrier chicks sampled in 2021. Ranks of chicks correspond to the hatching order in clutches (R1 being the first-hatched chicks, i.e., the oldest). Sample size (n), range (min–max), mean, and standard deviation (SD) are given for each sex and rank class.

	R1	R2	R3	R4	Total
Males					
n	8	6	11	1	26
Range	1–6	1–5	1–16	–	1–16
mean ± SD	2.5 ± 1.8	2.8 ± 1.7	3.6 ± 4.2	6.0	3.19 ± 3.06
Females					
n	12	12	3	2	29
Range	1–12	1–4	1–6	1–14	1–14
mean ± SD	3.2 ± 3.3	2.7 ± 1.1	3.0 ± 2.6	7.5 ± 9.2	3.24 ± 3.11
Total					
n	20	18	14	3	55
Range	1–12	1–5	1–16	1–14	1–16
mean ± SD	2.90 ± 2.77	2.72 ± 1.27	3.50 ± 3.88	7.00 ± 6.56	3.22 ± 3.10

Table 2
Summary of the sum of concentrations of pesticides in Montagu's harrier chicks sampled in 2021. Ranks of chicks correspond to the hatching order in clutches (R1 being the first-hatched chicks, i.e., the oldest). Sample size (n), range (min–max), mean, and standard deviation (SD) are given for each sex and rank class.

	R1	R2	R3	R4	Total
Males					
n	8	6	11	1	26
Range	734.75–3184.67	24.41–1641.10	583.33–4565.70	–	24.41–4565.70
mean ± SD	1323.74 ± 804.90	896.58 ± 635.15	1498.04 ± 1074.64	2113.18	1329.27 ± 897.82
Females					
n	12	12	3	2	29
Range	426.05–5501.34	634.65–4337.48	606.30–1144.86	487.53–7265.51	426.05–7265.51
mean ± SD	1788.35 ± 1334.14	1504.84 ± 1024.65	919.22 ± 279.69	3876.52 ± 4792.75	1725.14 ± 1536.36
Total					
n	20	18	14	3	55
Range	426.05–5501.34	24.41–4337.48	583.33–4565.70	487.53–7265.51	24.41–7265.51
mean ± SD	1602.51 ± 1150.52	1302.09 ± 940.78	1374.01 ± 980.37	3288.74 ± 3538.60	1538.00 ± 1279.40

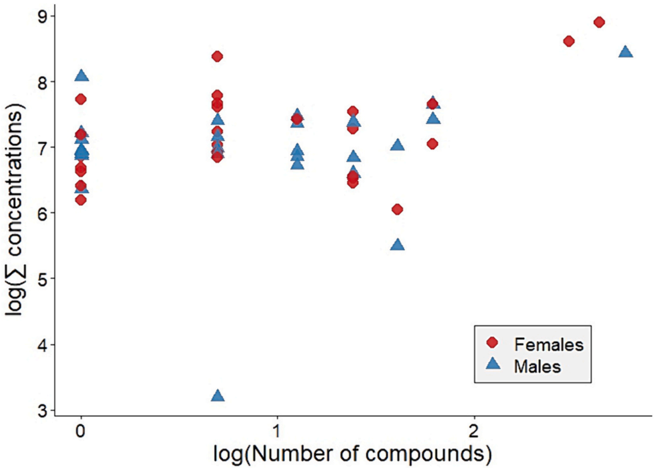

Fig. 2. Relationship between the number of pesticides detected and the sum of concentrations of pesticides in the blood of Montagu's harrier chicks. Both metrics were log-transformed for better visualization. Red dots represent females (n = 29) and blue triangles stand for males (n = 26).

biomonitoring schemes, as using an unsuitable method may lead to erroneous conclusions about the contamination levels.

4.2. *Effects of organic farming*

Despite the absence of effect of organic farming on the total concentrations of pesticides in chick's blood, we have showed that a higher proportion of organic farming around nests decreased the number of pesticides both at the field scale (300–500 m) and at a larger scale (1100–2200 m). This result is consistent with expectations; as organic farming practices exclude the use of synthetic pesticides, increasing the number of organic farming crop plots correspondingly reduces the pesticides found in the environment. In fact, in the present study, we detected some pesticides common to those reported in a recent study sampling small mammals in the study area (Fritsch et al., 2022), which is consistent with a dietary exposure route (17 out of the 28 compounds found in the present study; for more details see Fuentes et al., 2024). The lower number of compounds found in chicks from nests surrounded by higher proportions of organic farming at the field and larger scales, suggests that not only the direct environment of nests (soil and vegetation) is less contaminated but also that the prey hunted by parents in the close vicinity and brought to chicks is less contaminated. One way to properly investigate this hypothesis would be to quantify contamination levels in food pellets collected at nests. Another important habitat features in the nests' surroundings to consider would be hedgerows, as these semi-natural elements are known to increase small mammal abundance (Gelling et al., 2007) and to act as a natural barrier to pesticide drift (Langenbach et al., 2022). Thus, one may expect that higher proportions of hedgerows around nests may decrease the contamination levels linked to the reduction of pesticide drift.

Table 3
Summary of the effects of organic farming (in a zone with a radius of 300 m around the nest) on the number of pesticides detected (Nb. pesticides) and effects of sex and rank of Montagu's harrier chicks on Nb. pesticides and on the log-transformed sum of concentrations [log(∑ concentrations)], analysed using linear mixed-effects model (LMM) and generalized LMM fitted with a negative binomial distribution. Chi-squared statistics χ^2, df = degrees of freedom, and p = p-value, are given for each model. Estimates, standard errors, statistic values, and significance of each effect are provided in Supplementary material, Table S3.

Parameters	χ^2	df	p
Nb. pesticides			
% Organic farming	8.91	1	<0.01
Nb. pesticides			
Sex	0.00	1	0.98
Rank	4.77	3	0.19
Sex × Rank	2.36	3	0.50
log(∑ concentrations)			
Sex	1.99	1	0.16
Rank	3.75	3	0.29
Sex × Rank	4.48	3	0.21

Although chicks from a nest surrounded by a higher proportion of organic farms had less pesticides in their blood, the variability in pesticide contamination among nests was similar to that within nests (see previous section). In blackbirds (*Turdus merula*), habitat-related differences in metal contamination levels were attributable to both environmental/dietary contamination levels and foraging behaviours of parents (Fritsch et al., 2012). Montagu's harrier females generally bring small prey items, especially insects, to nestlings by the end of the rearing period, whereas males keep bringing most of the prey—generally voles—and hunt kilometres away from their nests, selecting their foraging area according to prey availability (Garcia and Arroyo, 2005; Krupiński et al., 2020). Thus, the chicks' diet includes prey not only from local foraging areas (≤2200 m) but also from larger distances. Moreover, dietary composition has been suggested to be the main driver of contaminant transfer, as some food taxa exhibit higher levels of contaminants but also differences in quality (i.e., levels of essential elements or proteins), which affects the uptake of contaminants during digestion and/or their metabolization (Fritsch et al., 2012). Insects and voles differ in their nutritional composition as voles are energy rich, whereas insects are richer in carotenoids, an antioxidant provided only by diet (Sternalski et al., 2010). As these antioxidants are involved in the detoxification processes, a higher uptake of insects, thus of antioxidants, may lead to a higher detoxification of pesticide compounds, therefore reducing the number and concentration of pesticides (Møller et al.,

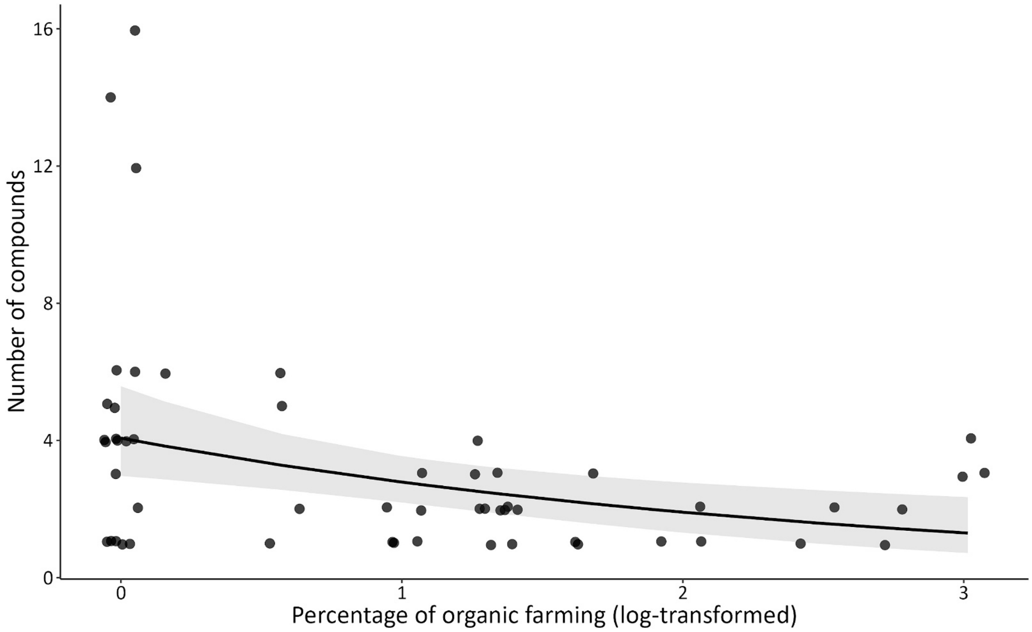

Fig. 3. Effect of organic farming (in a zone with a radius of 300 m around nests) on the number of pesticide compounds detected in Montagu's harrier chicks. The percentage of organic farming was log-transformed prior to model implementation. Dots were plotted with a small degree of random variation to the location of each point so that most chicks' values can be visualized. The line and the shading represent the predicted values and 95 % confidence interval from the generalized linear mixed-effects model fitted with a negative binomial distribution.

2000). Consequently, prey types may add contamination variability among broods depending on parents' hunting strategies and within the brood depending on which chick monopolizes the prey.

4.3. Effects of the sex and rank of chicks

In Montagu's harrier, females—being the larger sex—have an advantage over males in competition for larger food items within the brood (Arroyo, 2002). However, contrary to expectations, sex did not influence the number or the total concentrations of pesticides. Similarly for chick rank, we did not find an effect on contamination levels even in interaction with chick sex. However, our sample size for rank 4 chicks was limited to 3, mainly owing to the mortality of the latest hatched chicks, especially in years of low vole abundance, such as 2021 (V.B., unpublished data). Thus, our model lacks statistical power, which may explain why chick's rank did not have a significant effect despite the tendency observed. In fact, only half of the nests had more than two chicks at fledging (13 of 22 nests), whereas most had more than two laid eggs (20 of 22 nests), which indicates that the later-laid eggs and/or later-hatched chicks did not survive until sampling. For instance, in one of the nests monitored, only the first-hatched chick of the 5 laid eggs survived up to fledging. Smaller Montagu's harrier males that hatch later may have a higher probability to die from starvation (Arroyo, 2002). During the 2021 breeding season, low amounts of food resources were available, as reflected by the lower reproductive success in 2021 than in other years monitored in the study area (see Fuentes et al., 2023b for an overview of brood sizes in previous years). Thus, we cannot exclude the possibility that males that hatched later died before being sampled. Another explanation for the higher likelihood of death in younger chicks would be their pesticide load, assuming that there is a tendency of higher contamination levels in the last ranks. Indeed, the teratogenic effects of pesticides have been poorly investigated in wild birds, but some studies indicate that they reduce clutch sizes and increase chick mortality (Hernández et al., 2008; Lopez-Antia et al., 2015; Ortiz-Santaliestra et al., 2020). Thus, a contamination bias towards last eggs/younger chicks would lead to their hatching failure/premature death. In the present study, molecules showing teratogenic effects *in natura* were not tested for (organochlorines and polychlorinated biphenyls, Hernández et al., 2008; thiram, Lopez-Antia et al., 2015) or detected (tebuconazole and 2.4-D, Ortiz-Santaliestra et al., 2020) in fledglings. However, future studies should sample and analyse the pesticide content of unhatched eggs and younger chicks. Moreover, pesticides can lead to endocrine disruption with diverse consequences depending on the sex and on the cocktail of molecules involved such that males and females may differ in their sensitivity to pesticide accumulation (see Moreau et al., 2022 for review). An early death of males that hatched later in a brood may thus be attributable to both poor food conditions, their low ability to compete for food, and a potentially higher contamination load. If so, there should be a difference in the number of later-hatched females and males, biased towards females; however, we did not find a sex ratio bias in any of the rank classes. This contradicts an increased death rate of later-hatched males. However, differences in sex distribution among ranks should be properly investigated using, for example, DNA sexing of unhatched eggs and hatchlings because chicks cannot be visually sexed before they are 15 days old. Together with the quantification of pesticide loads of unhatched eggs, this would also constitute a promising approach to investigate teratogenic effects in farmland birds. An alternative explanation for the lack of effect of sex and rank is linked to the dominant–subordinate interactions among siblings. In fact, brood competition for food items may lead to unequal ingestion of pesticides among chicks belonging to the same nest. Therefore, we hypothesize that the most vigorous and aggressive chick, irrespective of its rank or sex, is the one monopolizing more and larger prey and thus displays the higher pesticide load. The role of brood competition in this context can be investigated through the examination of chick behaviour, either directly from video recordings or from behavioural metrics (e.g., the rate of beak attacks) taken during chicks' handling.

4.4. Limits and prospects

To our knowledge, this is the first study to report a high variability in the level of pesticide exposure among different chicks from the same nest. High intra-brood variability in contamination levels have been

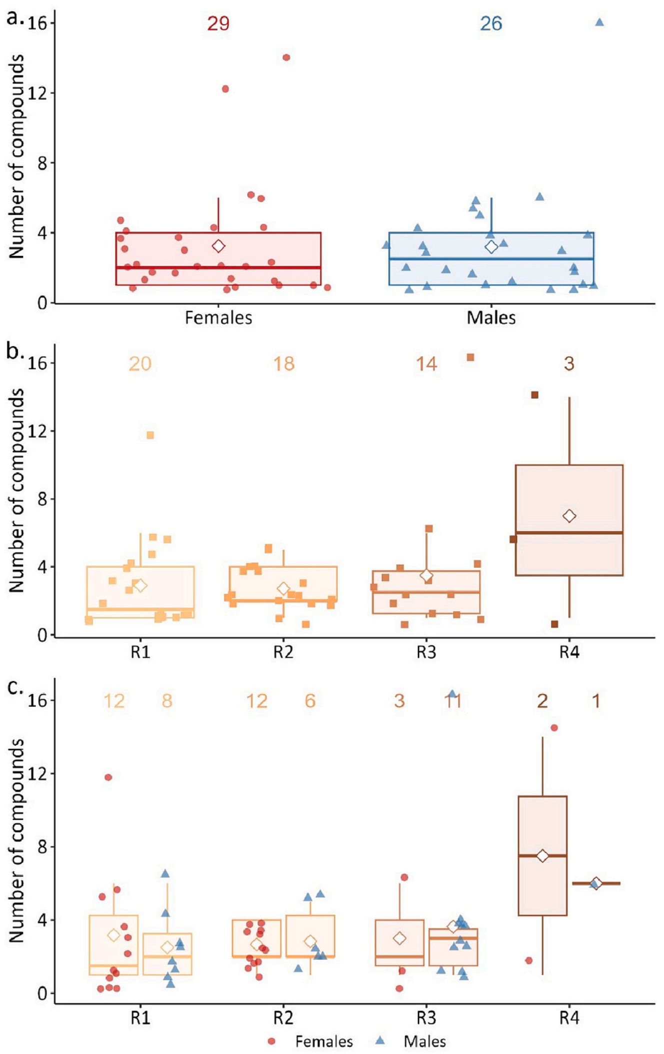

Fig. 4. Number of pesticides detected in Montagu's harrier chicks according to their sex (a), rank (b), and their interaction (c). The bottom and top lines of the boxes correspond to the first and third quartiles, middle line corresponds to the median, and whiskers correspond to the lower and higher values included in the 95 % confidence interval. All values were plotted with a small degree of random variation to the location of each point; therefore, they do not correspond to a round number. Blue triangles stand for males and red circles for females. Blank diamonds correspond to the mean; sample sizes are specified above the boxes.

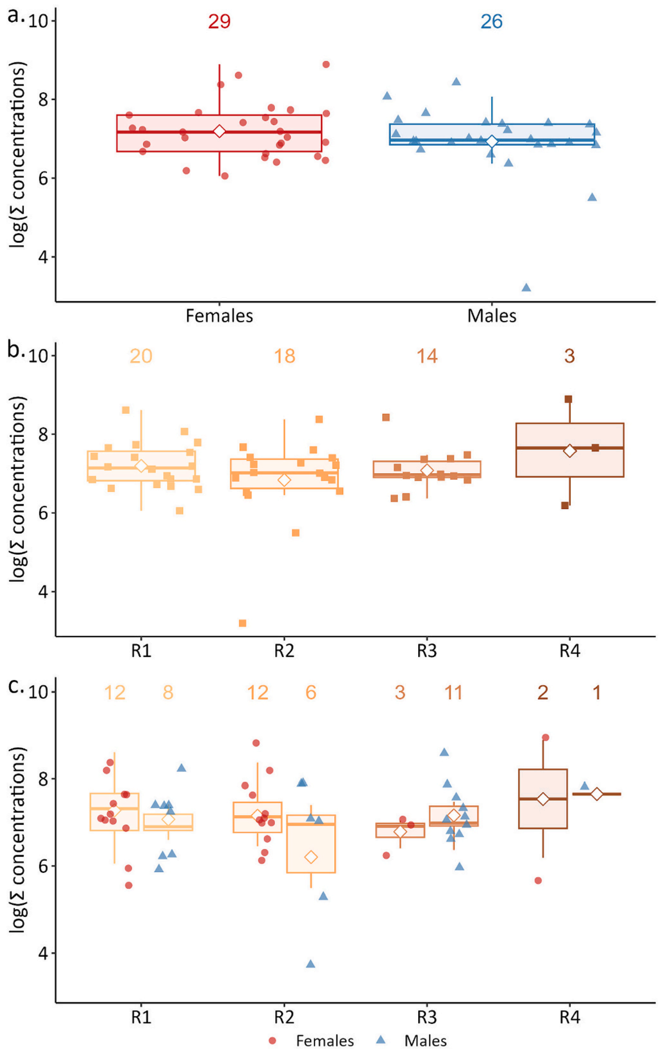

Fig. 5. Sum of concentrations of pesticides (log-transformed) in Montagu's harrier chicks according to their sex (a), rank (b), and their interaction (c). For legend details, see Fig. 4.

previously reported for heavy metals with contrasting results depending on the species and the contaminant studied (Janssens et al., 2002; Hofer et al., 2010). In addition, these investigations generally focused on eggs or feathers as biological matrices. Distribution of contaminants in biological tissues depends on their chemical properties and affinity with the tissue; thus, detection levels from different matrices are difficult to compare (Espín et al., 2016). Considering the present findings, biomonitoring schemes should carefully consider the model species, the contaminants involved, and the matrix sampled, to avoid misleading calculations of contamination levels. Moreover, it should be noted that the contamination levels reported here depend on the detection limits, which are method-dependent. Analytical methods differ in their sensitivity—a lower LOD would have increased the probability of detection, likely leading to a higher number of compounds detected. This should be considered whenever comparing contamination level variability among studies.

In the present study, we did not find the same pattern for the effect of organic farming on the two measures of contamination levels of chicks. Whereas the negative relationship between the proportion of organic farming and the number of compounds was clear, the link between organic farming and total concentrations was not statistically supported. This mismatch might arise from the fact that some nestlings with few pesticides had them in large concentrations. Moreover, high total concentrations do not necessarily indicate high toxicity to organisms as this will depend on the relative toxicity of each compound and on the presence of other compounds with potentially synergistic or antagonistic effects (Hernández et al., 2017). As no alternative to consider the complexity of such effects is available to date, the use of concentration addition is still widely applied, but results should be considered with caution.

5. Conclusions

Because the Montagu's harrier is at the top of the trophic chain and a specialist predator species of agricultural lands, studying its contamination with pesticides is particularly relevant as an indicator of larger contamination of the environment (*One Health* concept). The present study reveals that organic farming reduces the number of pesticides in Montagu's harrier chicks, which may have a beneficial effect on its population, as chemical inputs have been shown to drive farmland bird population decline across Europe (Rigal et al., 2023). Nevertheless, future work should investigate how pesticide cocktails are linked to the life-history traits of birds and affect population dynamics. Deciphering the effects of pesticides on birds' health status could potentially clarify the underlying mechanisms linking pesticide inputs and decline of farmland birds.

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CRediT authorship contribution statement

Elva Fuentes: Writing – original draft, Formal analysis, Conceptualization. **Jérôme Moreau:** Writing – review & editing, Supervision, Conceptualization. **Maurice Millet:** Writing – review & editing, Formal analysis. **Vincent Bretagnolle:** Writing – review & editing, Conceptualization. **Karine Monceau:** Writing – review & editing, Supervision, Funding acquisition, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.172778.

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Supplementary material

Organic farming reduces pesticide load in a bird of prey

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Table S1. Pesticides detected in the blood of 55 Montagu's harrier (*Circus pygargus*) chicks. Method to quantify pesticides was either liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) or automated thermal desorption gas chromatography coupled with tandem mass spectrometry (ATD-GC-MS/MS); LOD = limit of detection; LOQ= limit of quantification; N = number of individuals in which the compound was detected and if quantified (>LOQ) used to obtain mean, SD = Standard Deviation, and range (minimum–maximum), all concentrations are given in pg.mg^{-1} (=ng.g^{-1} = ppb).

Compound	Method[1]	LOD	LOQ	N	Mean ± SD[5]	Minimum	Maximum
Herbicides							
Bifenox	ATD-GC-MS/MS	0.0012	0.0038	10	<LOQ	-	-
Carbetamide	LC-MS/MS	0.0053	0.0176	1	29.75	-	-
Chloridazon	ATD-GC-MS/MS	0.0213	0.0709	17	121.02 ± 125.90	35.96	563.10
2,4-MCPA	ATD-GC-MS/MS	0.1579	0.5263	2	2020.12 ± 176.57	1895.27	2144.98
Mecoprop-P	ATD-GC-MS/MS	0.0500	0.1667	3	799.35 ± 369.40	409.93	1144.79
Metamitron	ATD-GC-MS/MS	0.0577	0.1923	8	25.01 ± 28.29	3.28	88.34
Oxadiazon	ATD-GC-MS/MS	0.0086	0.0286	1	71.68	-	-
Propyzamide	ATD-GC-MS/MS	0.0021	0.0071	1	339.07	-	-
Sulcotrione	LC-MS/MS	0.0021	0.0071	53	1111.78 ± 540.67	312.42	3184.67
Tebutam	ATD-GC-MS/MS	0.0526	0.1754	24	64.62 ± 49.77	13.81	180.24
Fungicides							
Boscalid	ATD-GC-MS/MS	0.0005	0.0016	3	1369.09 ± 819.48	791.50	2307.00
Carbendazim	LC-MS/MS	0.0042	0.0140	7	97.50 ± 81.21	0.216	258.29
Cyproconazole	ATD-GC-MS/MS	0.0192	0.0639	3	70.04 ± 69.82	13.65	148.13
Cyprodinil	ATD-GC-MS/MS	0.0011	0.0036	2	41.34 ± 42.91	11.00	71.68
Difenoconazole	ATD-GC-MS/MS	0.0359	0.1196	15	240.02 ± 317.74	31.68	1213.55
Dimethomorph	ATD-GC-MS/MS	0.0072	0.0242	3	241.49 ± 94.27	163.33	346.19
Dimoxystrobin	ATD-GC-MS/MS	0.0038	0.0128	2	171.79 ± 16.87	159.86	183.72
Epoxyconazole	LC-MS/MS	0.0027	0.0091	1	51.05	-	-

Flusilazole	ATD-GC-MS/MS	0.0144	0.0481	1	137.63	-	-
Myclobutanil	ATD-GC-MS/MS	0.0214	0.0714	1	142.12	-	-
Prochloraze	ATD-GC-MS/MS	0.0170	0.0568	1	1292.89	-	-
Quinoxyfen	ATD-GC-MS/MS	0.0048	0.0161	3	39.93 ± 38.73	9.73	83.59
Insecticides							
Bifenthrin	ATD-GC-MS/MS	0.0035	0.0116	1	18.46	-	-
Clothianidin	LC-MS/MS	0.0103	0.0344	3	929.30 ± 1268.76	189.92	2394.32
Cypermethrin	ATD-GC-MS/MS	0.0013	0.0042	2	204.34 ± 46.88	171.19	237.49
Indoxacarb	ATD-GC-MS/MS	0.0069	0.0231	3	<LOQ	-	-
Piperonyl butoxide	ATD-GC-MS/MS	0.0004	0.0015	5	36.30 ± 22.48	12.60	70.67
Thiacloprid	LC-MS/MS	0.0014	0.0048	1	87.48	-	-

Table S2. Main properties of pesticides detected in blood samples of Montagu's harrier nestlings. The DT50 ranges (detection time 50% = time to detect a 50% decrease in pesticide concentration) provided show the minimum and maximum values from field studies or from general literature (when field data were not available) and can vary greatly depending on the sources (for more details see Lewis et al., 2016). Model species corresponds to the birds for which the LD50 (lethal dose 50% = quantity of pesticide killing 50% of the test animals) was obtained: *Colinus virginianus* (Cv), *Coturnix japonica* (Cj), *Anas platyrhynchos* (Ap), and *Serinus canaria* (Sc). Main crops, DT50, Bird LD50, and model species were compiled from the Pesticide Properties DataBase (PPDB) of the University of Hertfordshire (http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm, accessed on 21 August 2023; Lewis et al., 2016). Type and mode of action were obtained from the Herbicide, Fungicide, and Insecticide Resistance Action Committees' online posters (available at https://www.hracglobal.com/, https://www.frac.info/, https://irac-online.org/ accessed on 21 August 2023). "Ban" corresponds to prohibition years in France; data obtained from legislative texts (available at https://www.legifrance.gouv.fr/ accessed on 21 August 2023). NA: not applicable when not considered as an active substance of plant protection products in Europe.

	Main crops	Ban	DT50 range (days)	Bird LD50 ($mg.kg^{-1}$)	Model species	Mode of Action
2,4-MCPA	Cereals, grass, linseed, asparagus, citrus	Yes	25	377	Cv	Auxin mimicking
Bifenox	Cereals, soybean, peanut, sugar beet, rice, sorghum, oilseed rape, sunflower	Yes	8.3–32.1	>2000	Cv	Inhibition of protoporphyrinogen oxidase
Bifenthrin	Ornamentals, sports fields, lawns	No (2019)	65–125	1800	Cv	Sodium channel modulation
Boscalid	Vegetables, soybean, fruits	Yes	196–312.2	>2000	Cv	Inhibition of succinate dehydrogenase
Carbendazim	Cereals, oilseed rape, beans	No (2014)	20–40	>2250	Cv	Tubulin polymerization
Carbetamide	Oilseed rape, vegetables, alfalfa, grape	No (2021)	8	>2000	Cv	Inhibition of microtubule organization
Chloridazon	Sugar beet, beet, onions, shallots, ornamentals	No (2018)	3–105	>2000	Cv	Inhibition of photosynthesis at PS II
Clothianidin	Corn, rice, orchards	No (2018)	13.3–1386	430	Cv	Competitive modulation of nicotinic acetylcholine receptor (nAChR)
Cypermethrin	Cereals, vegetables, oilseed rape, sugar beet, fruits, grassland	Yes	9.3–31.2	>9520	Ap	Sodium channel modulation

Cyproconazole	Cereals, vegetables, oilseed rape, sugar beet, fruits	No (2021)	62.1–501.2	94	Cv	Inhibition of demethylation
Cyprodinil	Cereals, fruits, ornamentals	Yes	11–98	>500	Ap	Inhibition of methionine biosynthesis
Difenoconazole	Cereals, vegetables, corn, cotton	Yes	20–265	>2150	Ap	Inhibition of demethylation
Dimethomorph	Fruits, grape, onions, cucurbits	Yes	34–54	>2000	Cv	Interference with cellulose synthase.
Dimoxystrobin	Wheat, oilseed rape	Yes	2–39	>2000	Cv	Inhibition at Quinone outer binding site
Epoxiconazole	Cereals, sugar beet, coffee, banana	No (2019)	52–226	>2000	Cv	Inhibition of demethylation
Flusilazole	Cereals, sugar beet, oilseed rape, fruits	No (2008)	63–240	>1590	Ap	Inhibition of demethylation
Indoxacarb	Corn, vegetables, fruits, cotton	No (2021)	4.9–7.5	73.5	Cv	Voltage-dependent sodium channel inhibition
Mecoprop-P	Cereals, sports fields, lawns	Yes	21	>500	Ap	Auxin mimicking
Metamitron	Sugar beet, beets	Yes	11.1	1302	Cj	Inhibition of photosynthesis at PS II
Myclobutanil	Turfs, ornamentals, orchards, vineyards	No (2021)	9–66	510	Cv	Inhibition of demethylation
Oxadiazon	Turfs, sports fields, ornamentals, vineyards	No (2018)	90–330	>2150	Cv	Inhibition of protoporphyrinogen oxidase
Piperonyl butoxide	NA	Yes	NA	NA	NA	Synergistic action
Prochloraz	Cereals, oilseed rape, fruits, turfs, avocado	No (2021)	28.6–245	662	Cv	Inhibition of demethylation
Propyzamide	Alfalfa, oilseed rape, beans, fruits, ornamentals	Yes	13.9–271.3	6578	Cj	Inhibition of microtubule assembly
Quinoxyfen	Cereals, grape, cucurbits, tomato	No (2019)	13–190	>2250	Cv	Inhibition of signal transduction
Sulcotrione	Cereals, corn, sunflower, oilseed rape, soybean, cotton	Yes	10.8–89.7	>1350	Ap	Inhibition of hydroxyphenyl pyruvate dioxygenase
Tebutam	Oilseed rape, sunflower, peanut, soybean, cotton	No (2002)	60	>5000	Ap	Inhibition of microtubule assembly
Thiacloprid	Vegetables, turfs, sod farms, landscape plants, ornamentals	No (2018)	5.95–16.8	35	Sc	Competitive modulation of nicotinic acetylcholine receptor (nAChR)

Table S3. Estimate, standard error (SE), statistic value (*z*), and significance (*p*) of each effect tested on the number of pesticides detected in Montagu's harrier chicks using generalized linear mixed-effects models fitted with a negative binomial distribution

Parameter	Estimate	SE	*z*	*p*
Intercept	1.40	0.16	8.72	< 0.001
% Organic farming (300 m)	-0.38	0.13	-2.98	< 0.01
Intercept	1.07	0.22	4.90	< 0.001
Sex M	-0.38	0.33	-1.15	0.25
Rank 2	-0.18	0.27	-0.65	0.51
Rank 3	0.09	0.44	0.20	0.84
Rank 4	0.25	0.38	0.65	0.52
Sex M × Rank 2	0.60	0.47	1.28	0.20
Sex M × Rank 3	0.39	0.55	0.71	0.47
Sex M × Rank 4	0.94	0.71	1.31	0.19

Table S4. Estimate, standard error (SE), statistic value (*t*), and significance (*p*) of each effect tested on the sum of concentrations of pesticides (log-transformed) in Montagu's harrier chicks using linear mixed-effects models

Parameter	Estimate	SE	*t*	*p*
Intercept	7.28	0.23	31.71	<0.001
Sex M	-0.21	0.36	-0.59	0.55
Rank 2	-0.13	0.32	-0.39	0.69
Rank 3	-0.48	0.51	-0.94	0.35
Rank 4	0.25	0.61	0.41	0.68
Sex M × Rank 2	-0.73	0.54	-1.35	0.18
Sex M × Rank 3	0.58	0.63	0.91	0.37
Sex M × Rank 4	0.34	1.04	0.33	0.74

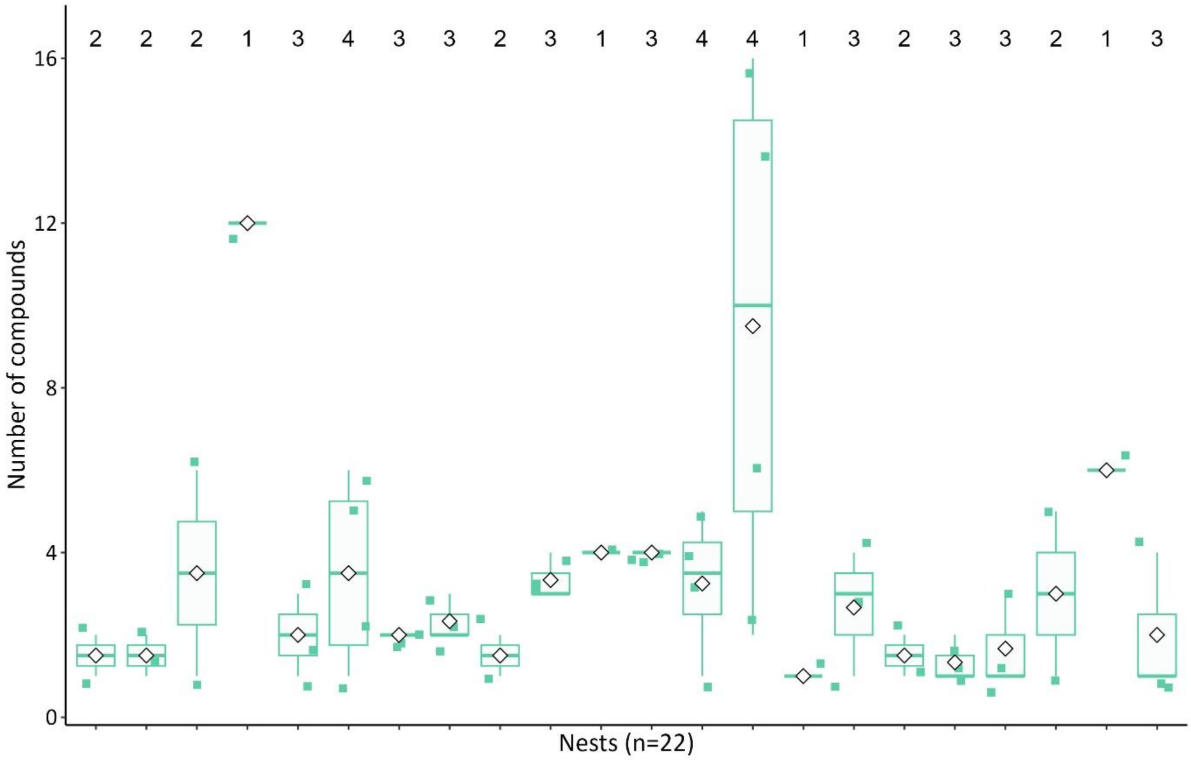

Figure S1. Variation in the number of pesticides detected in Montagu's harrier chicks according to the nest they belonged to. The bottom and top lines of the boxes correspond to the first and third quartiles, the middle line corresponds to the median, and the whiskers correspond to the lower and higher values included in the 95% confidence interval. All values were plotted with a small degree of random variation to the location of each point; therefore, they do not correspond to a round number. Blank diamonds depict the mean; sample sizes are specified above the boxes.

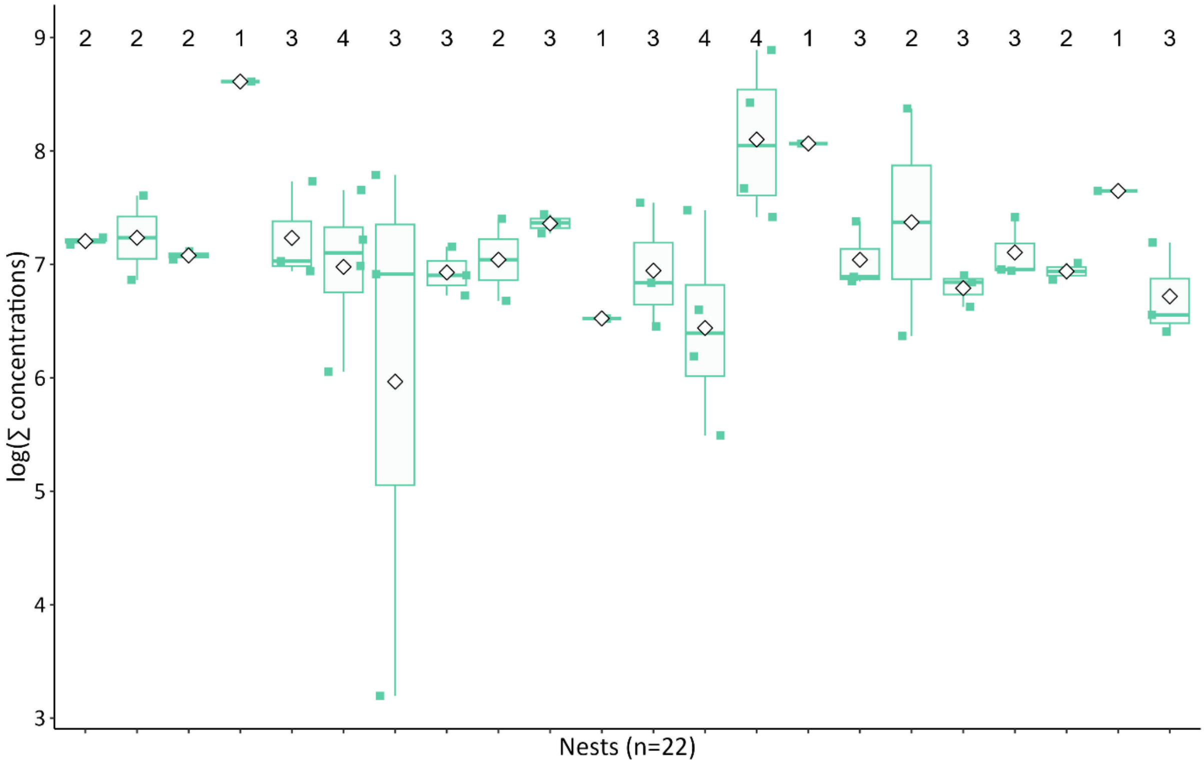

Figure S2. Variation in the sum of concentrations (log-transformed) of pesticides detected in Montagu's harrier chicks according to the nest they belonged to. For legend details, see Figure S1.

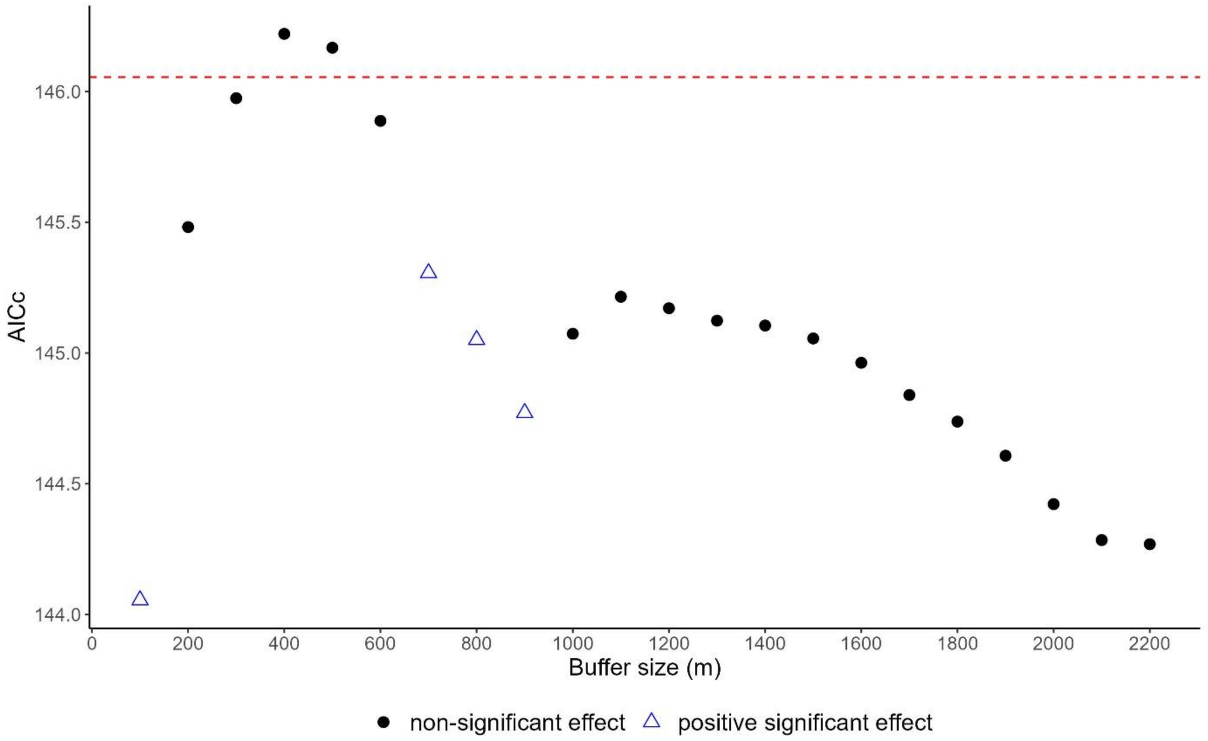

Figure S3. Model selection using AICc to assess the response of the total concentration (log-transformed) of pesticides detected in Montagu's harrier chicks to the effect of the percentage of organic farming around the nests at different distances (buffer sizes ranging from 100 m to 2200 m radius). AICc = Akaike Information Criterion corrected for small sample sizes. The red dotted line corresponds to the lowest AICc of candidate models + 2. Red triangles indicate a negative significant effect of organic farming (i.e., a negative model estimate and assorted 95% confidence interval not crossing 0). Black dots represent a non-significant effect of organic farming (i.e., the 95% confidence interval of model estimate crosses 0).

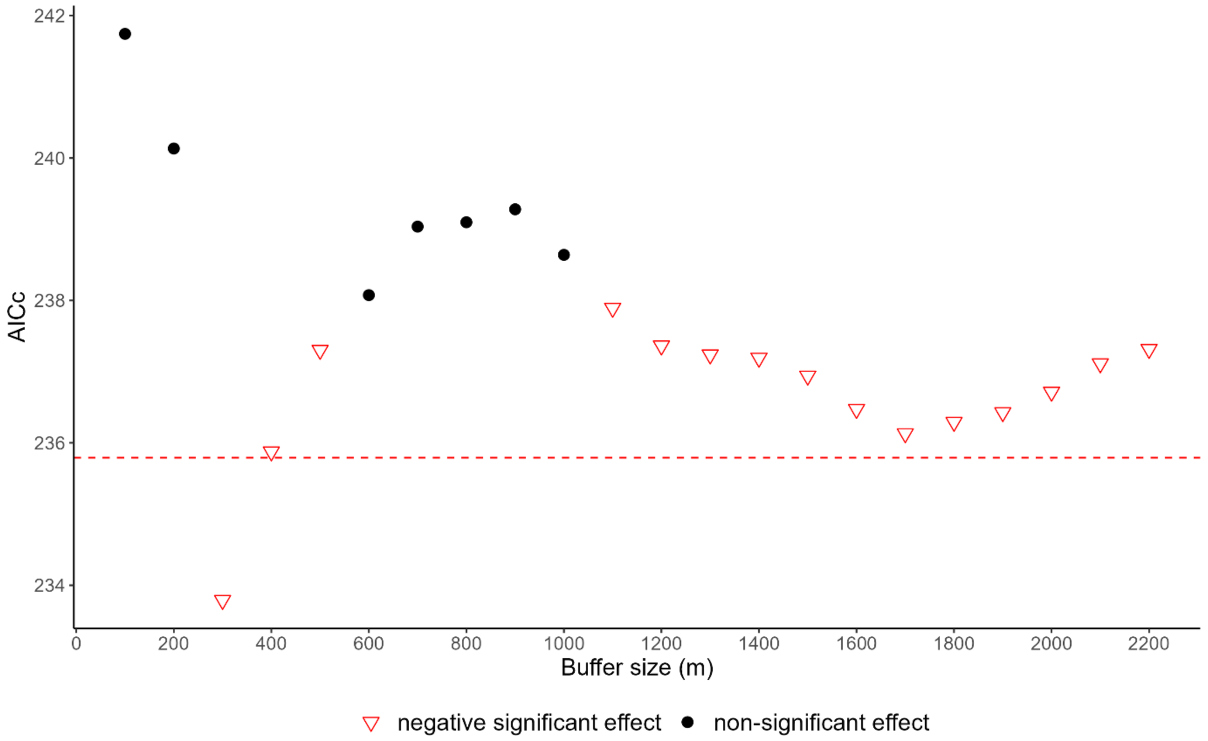

Figure S4. Model selection using AICc to assess the response of the number of pesticides detected in Montagu's harrier chicks to the effect of the percentage of organic farming around the nests at different distances (buffer sizes ranging from 100 m to 2200 m radius). For legend details, see Figure S3.

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Lewis, K.A., Tzilivakis, J., Warner, D., Green, A., 2016. An international database for pesticide risk assessments and management. Hum. Ecol. Risk Assess. 22, 1050-1064. https://doi.org/10.1080/10807039.2015.1133242

ARTICLE 4

FIRST EVIDENCE OF AGE-DEPENDENT DECREASES IN NON-PERSISTENT PESTICIDE MIXTURES IN NESTLINGS OF A FARMLAND RAPTOR

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First evidence of age-dependent decreases in non-persistent pesticide mixtures in nestlings of a farmland raptor

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ABSTRACT

Intensive use of synthetic pesticides in conventional agriculture may harm non-target organisms through sub-lethal effects on life-history traits. Farmland birds are exposed throughout their life cycle, but the fate of non-persistent pesticide mixtures in wild birds remains unknown. In this study, we investigated changes in pesticide contamination levels in Montagu's harrier (*Circus pygargus*) nestlings during their growth. In total, 35 chicks were sampled twice during the rearing period, and blood was tested for 116 pesticides to assess pesticide load through two proxies; the number of pesticides detected and the sum of pesticide concentrations. Body mass and tarsus length were also measured to estimate body condition. Across the two sampling times, nine herbicides, five insecticides and four fungicides were detected. Contamination levels decreased significantly with nestling age irrespective of sampling date, and there was no relationship between pesticide load and body condition. Moreover, concentrations of chlorpyrifos-methyl, fenpropidin, metamitron, picloram and S-metolachlor, all detected throughout the rearing period, were unrelated to any of the explanatory variables. However, ethofumesate concentrations decreased significantly with chick age. This study provides the first evidence that non-persistent pesticide mixtures can decrease with age in wild nestlings. This has implications for understanding how chicks are contaminated and provides new insights on pesticide fate within organisms.

1. Introduction

In agroecosystems, the intensification of agriculture has led to landscape homogenisation and increasing mechanisation and chemical inputs (Matson et al., 1997; Stanton et al., 2018). Among chemical inputs, pesticides are applied to prevent and control pests and weeds that may cause diseases or compete with crop plants, and include herbicides, fungicides, insecticides and rodenticides. Multiple pesticides are applied in fields simultaneously or sequentially, adsorbed by target and non-target species, and then supposed to be naturally degraded in the environment through a variety of processes, leaving by-product residues (Al-Mamun, 2017). However, the drift and persistence of some pesticides may lead to them remaining in soils and aquatic environments, possibly far from their application location, and even in untreated crops, sometimes years after their last application (Humann-Guilleminot et al., 2019; Wintermantel et al., 2020; Geissen et al., 2021). Consequently, even without direct exposure to some banned compounds, various species belonging to multiple trophic levels are contaminated with pesticide mixtures, highlighting the transfer of pesticides in all environmental compartments (Pelosi et al., 2021; Fritsch et al., 2022; Fuentes et al., 2023a, 2024a; Merleau et al., 2024). Some currently used pesticides are cleared from non-target organisms within a few hours to days (Bean et al., 2019; Pan et al., 2022). However, toxicokinetics is typically only conducted under controlled conditions for single compounds in one or a few model species, limiting knowledge extrapolation (Bean et al., 2019; Moreau et al., 2022; Pan et al., 2022). Little is known about the extent to which wild species are exposed to pesticides *in natura* (but see Bariod et al., 2024 for a captive *vs.* field comparison) and even less about how it changes over the season or during animal growth and development because very few studies have investigated variation in pesticide contamination levels over time in the field (but see Merleau et al., 2024). Thus, the fate of synthetic pesticide mixtures within wild organisms remains unknown, which is a concern because they are considered a major factor responsible for biodiversity decline, especially

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among farmland birds that have declined sharply across Europe (Geiger et al., 2010; Rigal et al., 2023). Indeed, whether currently used or banned, pesticides are known to have lethal and sublethal effects in non-target organisms such as wild birds, even at low doses (see Mitra et al., 2011; Moreau et al., 2022; Fritsch et al., 2024).

In wild birds, studies on heavy metals and persistent organic pollutants (POPs) reported differences in contamination levels of adults at different breeding stages, with higher concentrations from pre-laying to chick rearing periods (Nordstad et al., 2012), in nestlings depending on the growth stage (Lemmetyinen et al., 1982; Honda et al., 1986; Ackerman et al., 2011), and across the lifetime, with moderate changes in heavy metal concentrations (Bauerová et al., 2020). For nestlings, there can be a reduction in contamination levels with ageing, or the reverse, depending on the pollutant (Lemmetyinen and Rantamäki, 1980; Honda et al., 1986; Bustnes et al., 2013), U-shaped variation (Ackerman et al., 2011), or no variation with age (Monteiro and Furness, 2001; Bustnes et al., 2003). In fact, during nestling growth, pollutant concentrations may vary with a reduction in lipid content, and with distribution in growing tissues and feathers (Honda et al., 1986; Becker and Sperveslage, 1989). Fat tissues are rich in lipophilic pollutants, particularly those provided by the females through egg yolk (Charnetski, 1976; Mineau, 1982); therefore, the decline in lipid content during chick growth coupled with both an increase in tissue mass (muscle, liver) acting as storage and excretion in growing feathers, may reduce the circulating concentrations of pollutants (Charnetski, 1976; Goutner et al., 2001; Dauwe et al., 2006). This process, known as a dilution effect, is larger in faster-growing chicks (Goutner et al., 2001), although in some cases dietary intake of pollutants can exceed the dilution (Charnetski, 1976; Honda et al., 1986; Ackerman et al., 2011). Nevertheless, to the best of our knowledge, changes in contamination levels of multiple non-persistent (i.e., not included in POPs) pesticides in birds, and their associations with fat stores during growth, have not been investigated.

Montagu's harrier (*Circus pygargus*) is a migratory specialist raptor, feeding on voles and nesting on the ground in cereal crops. Chicks are particularly valuable as bio-indicators of local environmental contamination levels because they are exposed to pesticide mixtures during their development (Fuentes et al., 2024a). They can be contaminated through multiple pathways, notably contact (with the soil, vegetation and rain), inhalation (contaminated air) and the consumption of contaminated prey brought by parents. Another possible origin is the maternal transfer of compounds from adult females to eggs (e.g., Bellot et al., 2022).

In this study, we explored temporal changes in pesticide loads (using total pesticide number and the sum of their concentrations) in Montagu's harrier chicks and potential relationships between their contamination levels and a reduction in their lipid stores during growth. Chicks were blood-sampled twice during growth to assess pesticide loads for 116 compounds. This matrix was expected to reflect both recent contamination from a few hours to days, depending on the compounds (Espín et al., 2016; Katagi and Fujisawa, 2021), and concentrations in other tissues assuming an equilibrium between blood and soft tissues, as shown for some compounds (Bustnes et al., 2013; Espín et al., 2016). Besides the lack of studies on the redistribution of non-persistent pesticides between blood and other tissues, temporal variation in contamination levels within organisms seemingly vary according to both species and pollutant, making difficult to predict variations of non-persistent pesticide mixtures. Nevertheless, we predicted that pesticide contamination levels would decrease with chick age due to a dilution effect, which should be reflected by a positive relationship between contamination load and chick fat stores.

2. Material and methods

2.1. Study area and model species

2.1.1. Zone Atelier Plaine & Val de Sèvre (ZAPVS)

The ZAPVS study area is located in southwestern France (46°11′N, 0°28′W). The 450 km^2 area is covered mainly by intensive agricultural fields (Fig. S1 in Supplementary Materials), with cereal crops covering ~41% of the area under cultivation between 2009 and 2016 (Bretagnolle et al., 2018). Crops under organic farming (i.e., not using synthetic pesticides and fertilisers; Regulation EU, 2018/848) accounted for ~11% of the ZAPVS surface in 2018 (Fig. S1).

2.1.2. Montagu's harriers

Montagu's harriers are migratory raptors, and their reproduction has been monitored in the ZAPVS area since 1994 (Bretagnolle et al., 2018). They nest on the ground in cereal fields, generally laying up to six eggs (Arroyo et al., 1998). Incubation and rearing periods last 29 days and 30–35 days, respectively (Arroyo et al., 2007). Breeding success depends mainly on the availability of its main prey, the common vole (*Microtus arvalis*), although orthopterans and passerine birds are also preyed upon (Salamolard et al., 2000). In our study site, breeding success is ~2.05 fledglings per breeding attempt (Arroyo et al., 2004), with brood sizes varying between one and five nestlings (see Fuentes et al., 2023b for an overview of several years). Males have home ranges of ~14 km^2 in the study area (Salamolard, 1997) and provision incubating females and chicks, while females may contribute to food provisioning later in the rearing period, hunting close to the nest (Garcia and Arroyo, 2005). During winter, Montagu's harriers from the studied population are located in western Africa (Garcia and Arroyo, 1998).

2.2. Data collection

2.2.1. Ethical statement

Handling of chicks was performed under license from the Centre de Recherches sur la Biologie des Populations d'Oiseaux (CRBPO; Museum National d'Histoire Naturelle; licence #1308). All experiments were conducted following French guidelines for the ethical use of animals in research (APAFIS#18557–2019010822312199v2).

2.2.2. Sampling design

In 2018, Montagu's harrier nests were searched for, located and recorded using global positioning system (GPS) coordinates (Fig. S1). Nests were visited twice for blood sampling during the chick-rearing period (mean duration between samplings 10 ± 3 days). Morphometric estimation of age was performed *a posteriori* using wing length (Arroyo, 1995; calculator available at https://busards.com/index.php/Outils/biometry), thus chick age varied between 10 and 23 days (mean ± standard deviation: 16 ± 3 days) during the first visit and between 23 and 29 days (26 ± 2 days) during the second visit. Chicks were banded with a unique coded aluminium ring provided by the Museum National d'Histoire Naturelle de Paris (France) and sexed according to iris colour, brown for females and grey for males (Leroux and Bretagnolle, 1996) during the first sampling visit. During this visit, chicks were carefully handled in the shortest time possible to collect morphometric data and blood samples, before being placed back in the nest. The same procedure was followed during the second nest visit, providing blood samples from two different ages for each chick. Nestlings were sampled between mid-June and the end of July 2018. Although 81 chicks (24 nests) were monitored in 2018, blood samples for pesticide titration were only available for 35 (22 nests), which are included in the present study. No sex-ratio bias among chicks was observed in 2018 (36 females and 45 males; Binomial test, $p = 0.37$), even when considering only the 35 chicks studied (17 females and 18 males; Binomial test, $p = 1.00$).

2.2.3. Morphometric measurements

Nestlings were weighed using a Pesola 500 g spring scale (accuracy ± 5 g). Tarsus length was measured with a digital calliper (accuracy ± 0.1 mm). Wing and tail length were measured with a ruler (accuracy ± 1 mm). As the right limbs of chicks were measured twice at first and second visits while left limbs were measured twice only for the second visit, mean tarsus length and mean wing length were used in further analyses.

2.2.4. Scale mass index (SMI)

SMI is a residual body mass, calculated as follows (Peig and Green, 2009):

$$SMI_i = Mass_i \times \left(\frac{Mean\ tarsus\ length\ of\ the\ group}{Mean\ tarsus\ length_i}\right)^{b_{sma}}$$

where b_{sma} is the slope of the major axis regression of body mass (log-transformed) on the mean tarsus length (log-transformed), using the standard major axis method from the 'smatr' package (Warton et al., 2012) in R v.4.2.2 software (R Core Team, 2022). Males and females were separated to calculate SMI, accounting for expected differences in growth patterns. The SMI of chicks is assumed to reflect their lipid stores (Peig and Green, 2009) and is thus expected to decrease with age due to the pre-fledging reduction of body reserves to attain the optimum mass for flight (Mauck and Ricklefs, 2005; Wright et al., 2006).

2.2.5. Blood sampling

Individual blood samples (50 μL) were collected by puncturing the brachial vein using a sterile needle and heparinized capillaries. Each sample was collected in a plastic microcentrifuge tube, transported to the laboratory in a cooler (0–5 °C), and stored at −20 °C for further analyses.

2.3. Analytical methods

Following Rodrigues et al. (2023), 50 μL samples of whole blood (i.e., red blood cells and plasma) were used for multiresidue analyses. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and automated thermal desorption gas chromatography coupled to tandem mass spectrometry (ATD-GC-MS/MS) were performed to detect and quantify 116 compounds in multiple reactions monitoring detection mode for both instrumentations. These 116 compounds included herbicides, fungicides, insecticides, a safener that improves herbicide selectivity towards weeds rather than crop plants, and a synergist that enhances pesticide action, all among the most used in France. To the 104 compounds validated in Rodrigues et al. (2023), 12 compounds were added for multiresidue analysis after optimisation and validation. Limit of detection (LOD) and limit of quantification (LOQ) for each compound detected are provided in Supplementary Materials Table S1. LODs varied between 0.001 and 0.124 pg mg^{-1}, and LOQs varied between 0.004 and 0.436 pg mg^{-1}.

2.4. Statistical analyses

For each chick, contamination levels were estimated as the total number of pesticides detected in blood (N_{pesti}) and the total sum of pesticide concentrations ($\sum$Conc). These metrics are commonly used in ecotoxicology studies to reflect levels of contamination (Jenssen et al., 2010; Tartu et al., 2014; Fritsch et al., 2022; Fuentes et al., 2024b). N_{pesti} and $\sum$Conc were correlated (Pearson's correlation test $\rho = 0.60$, 95% confidence interval = 0.45; 0.73), however, the presence of compounds (i.e., the number) and their quantity (i.e., the concentrations) might have different dynamics over time, thus we included both metrics in further analyses. Neither of the variables satisfied normality and homoscedasticity, therefore prior to model implementation, $\sum$Conc was square root-transformed and used as a response variable in a linear mixed effect model (LMM). Meanwhile, N_{pesti} was used as a response variable in a generalised linear mixed-effects model fitted with a Poisson distribution (PGLMM). For all models described hereafter, we used the identity of chicks nested within the nest identity as random effects (chicks were sampled twice and may belong to the same nest).

To investigate changes in contamination levels with chick age and relationship with chick lipid stores, we constructed one LMM ($\sum$Conc as response variable) and one PGLMM (N_{pesti} as response variable) using sampling date (in Julian days), age (days) and SMI (grams) of chicks as explanatory variables. Sampling date is relevant because any environmental (contamination levels, weather) and/or phenotypic (breeding onset) variation during the season might explain within-individual temporal patterns. Although SMIs of chicks are expected to decrease with age (Fig. S2 in Supplementary Materials), these variables were not included as interacting effects in the model because this led to multicollinearity (checked using Variance Inflation Factor, VIF). We did not include the sex of nestlings in models as contamination levels did not differ between males and females (Wilcoxon rank sum test: W = 753, $p = 0.10$ for the sum of pesticide concentrations, and W = 618, $p = 0.95$ for the number of pesticides detected), in line with previous results on Montagu's harrier chicks of the same population (Fuentes et al., 2024b). When a pesticide was detected during both sampling occasions, its temporal variation was investigated through LMM using its concentration (square root-transformed) as a response variable and using sampling date, age and SMI of chicks as explanatory variables. In all cases, the significance of each effect within the model was assessed using likelihood-ratio Chi-square tests (Fox and Weisberg, 2019). All analyses were performed in R v.4.2.2 software (R Core Team, 2022) using the packages 'car' (Fox and Weisberg, 2019) for VIF checking and likelihood-ratio Chi-square tests, 'DHARMa' (Hartig, 2022) to check model residuals, and 'lme4' (Bates et al., 2015) to implement mixed-effects models.

3. Results

In total, 18 pesticides were detected (i.e., concentrations > LOD) in blood samples from 35 chicks (70 blood samples), including 9 herbicides, 5 insecticides and 4 fungicides (Fig. 1 and Table S1 in Supplementary Materials). Chicks had on average (mean ± standard deviation) 5.60 ± 1.52 pesticides in their blood (N_{pesti}) and total concentrations of 980.97 ± 594.31 pg mg^{-1} ($\sum$Conc) during the first sampling. At the second sampling, mean N_{pesti} was 3.54 ± 2.55 and mean $\sum$Conc was 580.00 ± 735.34 pg mg^{-1}. Contamination levels of chicks decreased with increasing age, with the effect of age significant for both N_{pesti} and $\sum$Conc (Table 1; Fig. 2; Table S2 in Supplementary Materials). Neither N_{pesti} nor $\sum$Conc were influenced by the sampling date or SMI of chicks (Table 1 and Table S2, Figs. S3 and S4 in Supplementary Materials).

Seven pesticides were detected in the first samples alone, compared with five in second samples alone, and six in both samples (Fig. 1; Table S1). The concentrations of five of the pesticides present at both sampling occasions (chlorpyrifos-methyl, fenpropidin, metamitron, picloram, S-metolachlor) were not influenced by any of the explanatory variables (Table S3 and Fig. S5 in Supplementary Materials). Ethofumesate concentrations were significantly decreased with chick age, while sampling date and chick SMI had no significant effect (Table S3).

4. Discussion

The results of the present study provide the first evidence of a reduction in both the number and total concentrations of non-persistent pesticide mixtures with age in wild farmland bird nestlings. However, we did not find here a relationship between pesticide contamination levels in chick blood and body condition, suggesting that a mechanism other than a reduction in fat stores is responsible for the lower contamination levels as chicks age. When assessing the concentrations of pesticides present throughout chick growth, we observed a reduction

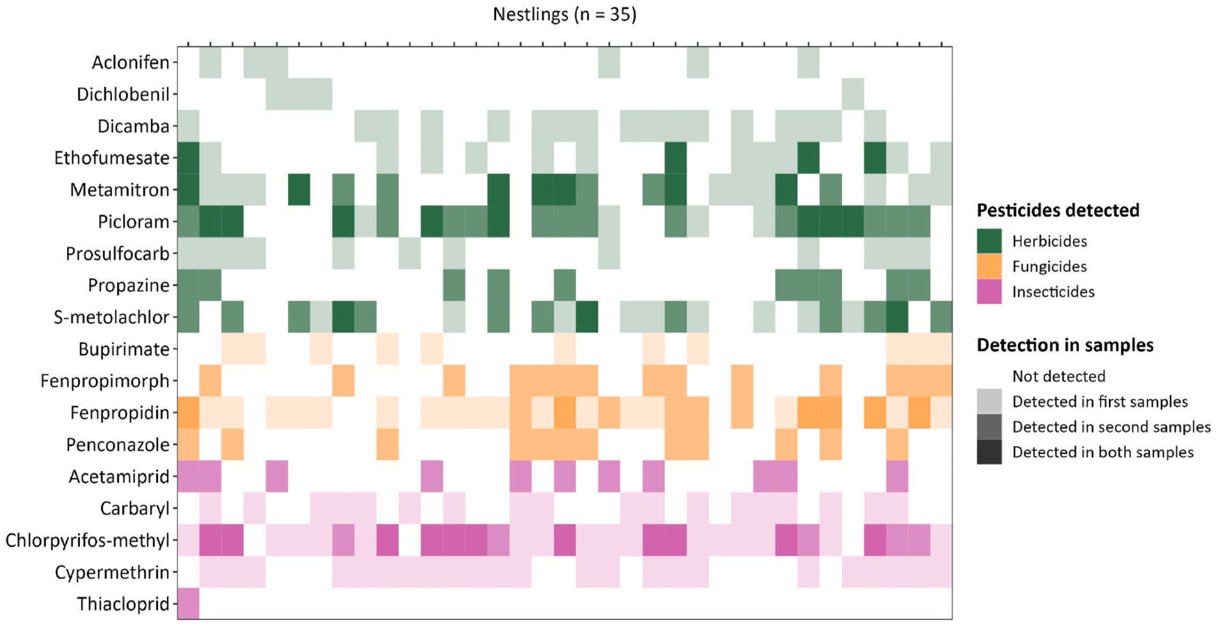

Fig. 1. Pesticides detected in Montagu's harrier (*Circus pygargus*) chicks of 10–23 days old in first samples and 23–29 days old in second samples. Columns represent chicks and rows represent pesticides. Each coloured cell (green for herbicides, orange for fungicides and pink for insecticides) indicates a detected pesticide, and shading levels indicate if detection occurred in first or second samples only, or both.

Table 1
Effects of sampling date (in Julian days), age (days) and body condition (grams) of Montagu's harrier chicks on pesticide contamination levels. The statistical significance of each variable was assessed by an analysis of deviance based on likelihood-ratio Chi-square tests (statistical values, degrees of freedom and *p*-values). Tests were performed on a linear mixed-effects model (LMM) for the square root-transformed sum of concentrations ($\sum$Conc) and on a generalised LMM fitted with a Poisson distribution (PGLMM) for the number of pesticides detected (N_{pesti}). Significant effects ($p < 0.05$) are depicted in bold.

Variables		χ^2	df	*p*
N_{pesti}				
	Sampling date	0.027	1	0.868
	Age	**5.573**	**1**	**0.018**
	SMI	0.141	1	0.707
$\sum$Conc				
	Sampling date	0.002	1	0.964
	Age	**7.958**	**1**	**0.005**
	SMI	0.370	1	0.543

with ageing only for ethofumesate, indicating that the reduced contamination loads with age are mainly attributable to the clearance of the seven pesticides detected only in younger nestlings, despite the detection of five other pesticides in older nestlings.

Although a dilution effect may be expected to occur during chick growth, we did not detect any link between contamination levels and body condition, despite a significant decrease in pesticide loads with aging. However, half of the compounds detected were not particularly lipophilic, as reflected by their octanol-water partition coefficients (log P values < 3; see Table S1 in Supplementary Materials). They might thus have a lower affinity for fat tissues and respond differently from POPs such as dichlorodiphenyltrichloroethane (DDT), known for its ability to be stored in fat tissues including egg yolk (log P = 6.91; see Blus, 2011), except cypermethrin that may bioaccumulate in fatty tissues (log P > 5; EPA, 2012). This may explain the lack of a relationship between pesticide load and body condition. Nonetheless, our study suggests that younger nestlings have a higher pesticide load that may be due to maternal detoxification into eggs (e.g., Bargar et al., 2001; Bellot et al., 2022), and during the latter part of the rearing period, the detoxification capabilities of chicks might allow decreased pesticide loads. Indeed, pesticide intake from the diet remains steady, as food provisioning is not likely to be decreased during the nestling period, but rather increased

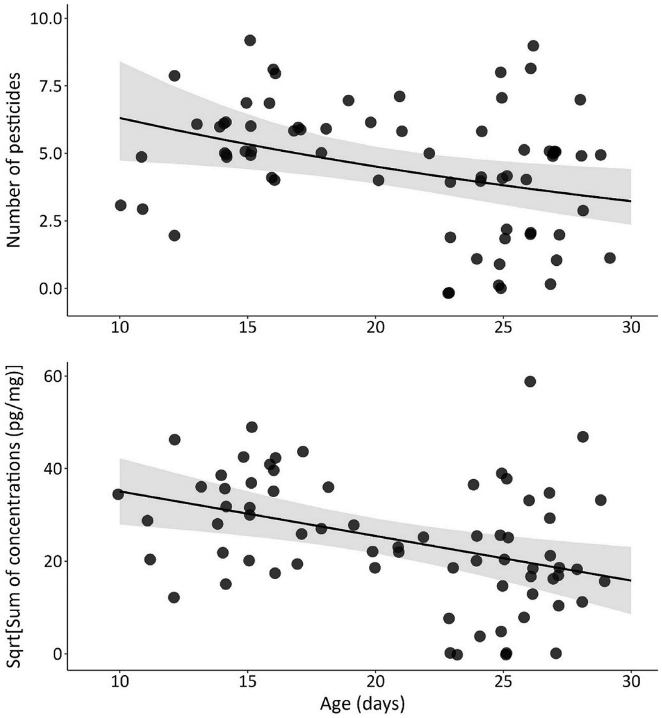

Fig. 2. Age-dependent decrease in pesticide contamination levels, both the number of pesticides (top) and the sum of pesticide concentrations (bottom), in Montagu's harrier (*Circus pygargus*) chicks. Lines and shading represent the values predicted for the effect of chick age and 95% confidence intervals from the linear mixed-effects model (LMM) fitted with a Poisson distribution (number of pesticides) and the LMM (sum of concentrations square root-transformed).

(Kitowski, 2003; Wieringa et al., 2019). Therefore, a higher pesticide excretion rate is needed in Montagu's harrier nestlings to result in the lower contamination levels observed. For instance, in Japanese quail chicks, the activity of esterases (enzymes involved in organophosphate detoxification) increased with age, indicating a higher capacity of synthesis of enzymes with chick ageing (Narváez et al., 2016). Studies on the development of detoxification mechanisms during bird growth are scarce, but they suggest that embryos and hatchlings have lower enzymatic activities, resulting in older nestlings having stronger metabolic and excretion capacities (Veini et al., 1986; Liu et al., 2019; Caviedes-Vidal and Karasov, 2001). Early-life changes in the gut microbiota might also explain the reduction in contamination levels with age because it is involved in detoxification processes (Grond et al., 2018; Liu et al., 2020) and displays a strong temporal variation during bird development (Teyssier et al., 2018; Zhu et al., 2021). Overall, temporal variation in pesticide mixtures in Montagu's harrier chicks is probably governed by the ontogenetic development of physiological mechanisms not explored herein that increase metabolic potential and counterbalance pesticide uptake.

Although a general trend in the reduction of pesticide load with age was observed, single pesticides might differ from one another in their temporal dynamics within individuals. Compounds detected in both samples may indicate the persistence of maternally transferred molecules in the organism (from fat tissues then delivered in blood) and/or permanent exposure during the rearing period. If the former was correct, the six compounds detected at both sampling periods should decrease with chick ageing, but no trends in concentration changes with age were observed for five of them (Fig. S5), which indicates an absence of metabolisation/detoxification and/or continuous high-level contamination of chicks with these pesticides throughout growth. The fungicide fenpropidin and the insecticide chlorpyrifos-methyl detected at both sampling periods may have been heavily used on cereal crops before sampling nestlings as cereals are predominant crops in the study area (Table S1; Figs. S1 and S6). Ethofumesate and metamitron are two herbicides that can be applied to seed carriers of industrial and fodder beet in the study area. However, only very few fields of these crops are present in the study area, which questions the origin of the contamination of nestlings with these substances. The herbicides picloram and S-metolachlor can be applied to oilseed rape and maize, respectively, both of which are major crops in the study area, covering 8.3% and 9.6%, respectively (Bretagnolle et al., 2018; Fig. S1), with treatment periods spanning part of the nestling rearing period (Fig. S6). Pesticide treatments may have resulted in direct exposure during application, through particle drift, *via* contact with adults and/or due to persistence of pesticides within these crops (plants and/or soil), resulting in contaminated eggs and nestlings and/or prey (none of these pathways are mutually exclusive). Metamitron, S-metolachlor and metabolites of chlorpyrifos-methyl have been detected in hairs of small mammals in the study area, supporting repeated exposure through the diet, while ethofumesate, fenpropidin and picloram were not searched for (Fritsch et al., 2022). Additionally, as males can hunt several kilometres away from the nest, crops outside the study area may influence the pesticide contamination of nestlings, which may result in a mismatch between the pesticides detected and the predominant crops in the study area (Fuentes et al., 2024a).

On the other hand, the detection of some compounds only in first samples might reflect early contamination, possibly through eggs, while detection only in second samples might reflect food or contact-mediated contamination later in the rearing period. For instance, dichlobenil and carbaryl, banned in France since 2008 and 2007, respectively (Fig. 2 and Table S1 in Supplementary Materials) were detected only in first samples, similar to the highly lipophilic cypermethrin, supporting the aforementioned explanation. The maximum reported half-life in soil (DT50, the time taken to reduce by 50% the dose applied) is six months for dichlobenil and 329 days for carbaryl (Lewis et al., 2016), while chicks were sampled ten years after they were banned. Thus, they could have been ingested by females in wintering areas, transferred to eggs, found in nestlings only in the first weeks post-hatching, and then excreted and/or stored in their growing tissues or feathers. Nonetheless, we detected propazine, a herbicide prohibited in France since 2002, in second samplings. Moreover, in a previous study on Montagu's harrier chicks aged ~26 days old from the same population, banned molecules were also detected (Fuentes et al., 2024a). This suggests either that maternally transferred compounds persist until chicks fledge (notably in liver and fat tissues) and are released in the bloodstream during the growing period, and/or that there is fraudulent use of such pesticides (Fuentes et al., 2024a). We cannot discount this last scenario based on detection of propazine only in second samplings, and because our first samplings probably occurred too late ($\geq$10 days post-hatching) to reflect only maternal transfer without a dietary input. Titration of pesticides in unhatched eggs of clutches could prove interesting because it might provide information on compounds deposited in eggs before chick hatching.

Regarding the five pesticides detected only in older nestlings, food or contact-mediated contamination is supported for penconazole, acetamiprid and thiamethoxam, as these have also been detected in small mammals and other environmental compartments in the study area (Wintermantel et al., 2020; Pelosi et al., 2021; Fritsch et al., 2022), while propazine has not been detected (Fritsch et al., 2022) and fenpropimorph was not investigated. Pesticide titration in food pellets collected at nests could provide novel insight into dietary contamination pathways.

Although the within individual temporal pattern is quite clear for Montagu's harrier chicks, the generality of our findings is complicated to assert for other species. Additionally, for a given species, the population studied might influence pesticide temporal patterns, not only due to differences in environmental pesticide loads, but also differences in diet and ecology. Indeed, as suggested previously (Fuentes et al., 2023b; 2024b), alternative prey in the diet of Montagu's harriers might be important due to differences in nutritional composition such as carotenoids and other antioxidants involved in detoxification processes (Møller et al., 2000; Sternalski et al., 2012), which could affect pesticide uptake and detoxification. Thus, additional studies are needed, including dietary and metabolic analyses, to better understand the fate of pesticide cocktails within organisms, and more broadly within the environment.

5. Conclusion

The present study revealed a reduction in both the number of pesticides detected and the sum of pesticide concentrations in the blood of wild nestlings of a farmland raptor with increasing age. Although the mechanisms leading to decreased contamination levels remain unclear, this provides a first step in understanding the fate of non-persistent pesticide mixtures within wild birds. The next step is to determine if these contamination levels can impact the development of Montagu's harrier nestlings, as this might unveil the factors underpinning the decline of this farmland bird.

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CRediT authorship contribution statement

Elva Fuentes: Writing – original draft, Visualization, Formal analysis, Conceptualization. **Jérôme Moreau:** Writing – review & editing, Supervision, Conceptualization. **Maurice Millet:** Writing – review & editing, Investigation, Formal analysis. **Vincent Bretagnolle:** Writing – review & editing, Conceptualization. **Karine Monceau:** Writing –

review & editing, Supervision, Investigation, Funding acquisition, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.120179.

Data availability

Data will be made available on request.

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SUPPLEMENTARY MATERIALS

First evidence of age-dependent decreases in non-persistent pesticide mixtures in nestlings of a farmland raptor

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Table S1. Pesticides detected in the blood of 35 Montagu's harrier (*Circus pygargus*) chicks in 2018. Analytical methods (GC = ATD-GC-MS/MS; LC = LC-MS/MS), limits of detection (LODs) and limits of quantification (LOQs) in pg.mg^{-1} are given for the 18 compounds detected. Log P = octanol-water partition coefficient log-transformed, from the Pesticide Properties Database (PPDB; http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm, accessed on 11 September 2024). The main crops of application concern uses stated in the French marketing authorisations (or former authorisations) of the phytopharmaceutical products containing the mentioned active substance, and were obtained from https://ephy.anses.fr/, accessed on 11 September 2024 (the list is not exhaustive and focuses on crops potentially present in the study area, those not present are indicated by * and include fruits, ornamentals and non-cropped areas). The number of detections (N) for the compounds, in total (max 70 samples) or for first and second samplings (max 35), are also indicated. Cells are coloured blue when pesticides were detected during both samplings. The years substances were banned are in italics if occurring after the nestling sampling period.

Pesticide type Compound	**Method**	**LODs**	**LOQs**	**Log P**	**Main crops**	**Banned in France**	**N total**	**N first samples**	**N second samples**
Herbicides									
Aclonifen	GC	0.044	0.147	4.37	Cereals, sunflower, maize, leguminous plants		6	6	-
Dicamba	GC	0.124	0.436	-1.80	Cereals, maize		17	17	-
Dichlobenil	GC	0.015	0.051	2.70	*	2008	4	4	-
Ethofumesate	GC	0.007	0.024	2.70	Seed carrier of industrial and fodder beet, forage and turf grasses		19	15	4
Metamitron	GC	0.058	0.192	0.85	Seed carrier of industrial and fodder beet		28	16	12
Picloram	GC	0.027	0.090	-1.92	Oilseed rape		32	12	20
Propazine	GC	0.070	0.380	3.95	Maize, sorghum	2002	10	-	10
Prosulfocarb	GC	0.098	0.358	4.48	Cereals, oilseed poppy		12	12	-

S-metolachlor	GC	0.010	0.032	3.05	Maize, sunflower, peas, sorghum, soybean	*2024*	25	12	13
Fungicides								-	-
Bupirimate	GC	0.022	0.075	3.68	*		11	11	-
Fenpropidin	GC	0.007	0.025	2.90	Cereals, seed carrier of industrial and fodder beet		34	23	11
Fenpropimorph	GC	0.003	0.009	4.50	Cereals	*2019*	14	-	14
Penconazole	GC	0.011	0.039	3.72	Grapevine		12	-	12
Insecticides								-	-
Acetamiprid	LC	0.010	0.034	0.80	Cereals	*2018*	11	-	11
Carbaryl	GC	0.028	0.102	2.36	Cereals	2007	18	18	-
Chlorpyrifos-methyl	GC	0.028	0.093	4.00	Cereals, grapevine	*2020*	43	27	16
Cypermethrin	GC	0.001	0.004	5.55	Maize, sunflower, cereals, sorghum, peas		23	23	-
Thiamethoxam	LC	0.012	0.041	-0.13	Maize, sorghum, grapevine	*2018*	1	-	1

Table S2. Summary of the effects of sampling date (Julian days), chick age (days) and scale mass index (SMI, grams) on contamination levels in nestling blood. N_{pesti} (total number of pesticides) was used as the response variable in PGLMM (z statistics); ∑Conc (total sum of pesticide concentrations in $pg.mg^{-1}$, square root-transformed) was used as the response variable in LMM (t statistics). Significant effects (*p* <0.05) are depicted in bold.

Variables	**Estimate**	**SE**	**Statistics (z or t)**	***p***
N_{pesti}				
Intercept	3.015	2.965	1.017	0.309
Date	-0.002	0.011	-0.166	0.868
Age	**-0.036**	**0.015**	**-2.361**	**0.018**
SMI	-0.002	0.005	-0.376	0.707
∑Conc				
Intercept	34.779	44.817	0.776	0.443
Date	-0.010	0.229	-0.045	0.964
Age	**-0.914**	**0.324**	**-2.821**	**0.007**
SMI	0.046	0.075	0.608	0.545

Table S3. Effects of sampling date (Julian days), age (days) and body condition (grams) of Montagu's harrier chicks on pesticide concentrations. The number of nestlings with concentrations quantified at both samplings and the total number of nestlings with concentrations >LOD for each molecule are in brackets. The significance of each variable was tested using likelihood-based chi-square tests (statistical values, degrees of freedom and *P*-values). Tests were performed on linear mixed-effects models (LMMs) for the square root-transformed concentrations of each pesticide. Significant effects (p <0.05) are depicted in bold.

Variables	**χ^2**	**df**	***p***
Chlorpyrifos-methyl (11/32)			
Sampling date	0.137	1	0.711
Age	0.252	1	0.616
SMI	2.930	1	0.087
Ethofumesate (4/15)			
Sampling date	0.034	1	0.853
Age	**5.693**	**1**	**0.017**
SMI	2.093	1	0.148
Fenpropidin (6/28)			
Sampling date	0.001	1	0.979
Age	3.289	1	0.070
SMI	2.785	1	0.095
Metamitron (7/21)			
Sampling date	0.000	1	0.995
Age	0.482	1	0.487
SMI	0.034	1	0.853
Picloram (8/24)			
Sampling date	0.074	1	0.785
Age	1.904	1	0.168
SMI	0.511	1	0.474
S-metolachlor (3/22)			
Sampling date	0.013	1	0.910
Age	0.000	1	0.985
SMI	1.095	1	0.295

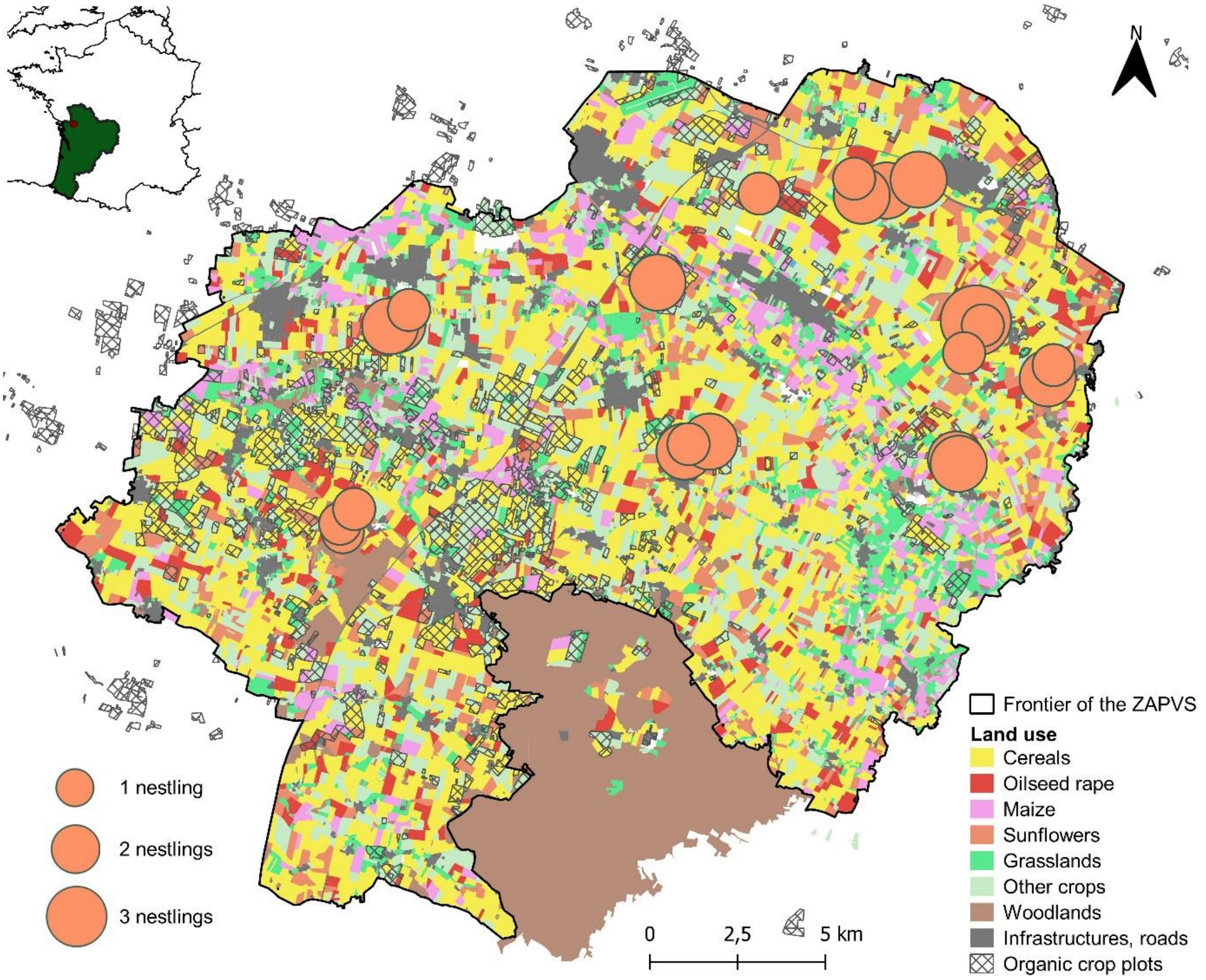


Figure S1. Geographical distribution of the 22 Montagu's harrier nests included in the study, and land uses in the Zone Atelier Plaine & Val de Sèvre (ZAPVS) area in 2018. In total, 35 chicks were sampled. Dot size is proportional to the number of nestlings sampled per nest (maximum 3 nestlings). Land uses were determined in 2018 by our team's fieldworkers driving through the study area. The map was thus created using data from our GIS database and the software QUANTUMGIS 3.16.5 geographical information system (available at: http://qgis.osgeo.org, accessed on 11 September 2024).

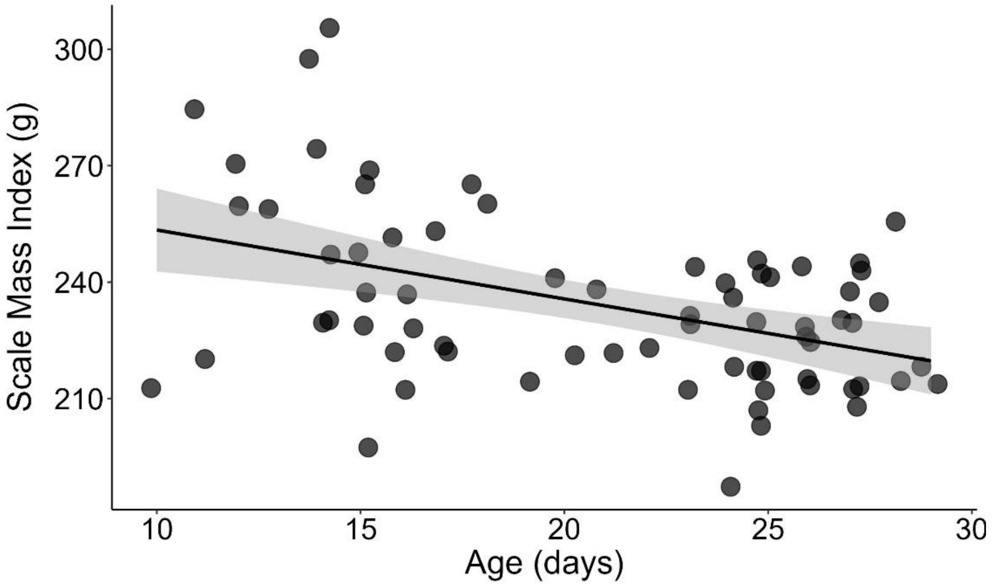


Figure S2. Decreasing scale mass index (SMI, in grams) of Montagu's harrier chicks with increasing age. The line and shading represent the values predicted for the effect of chick age and the 95% confidence interval from the LMM.

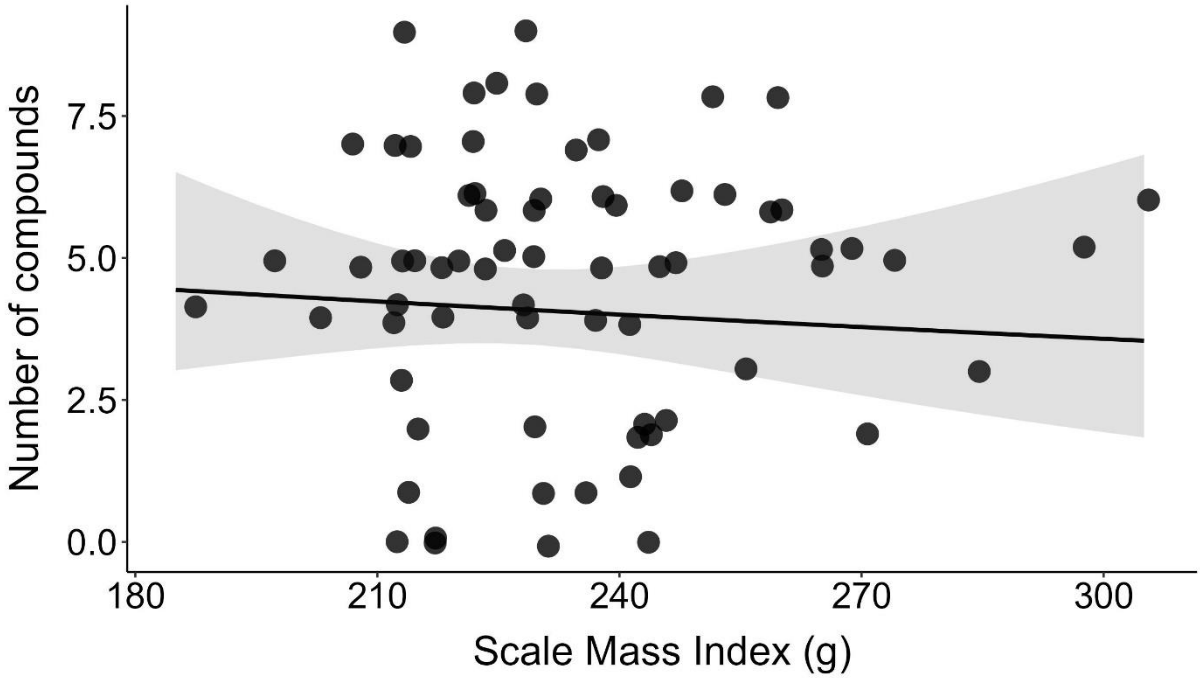


Figure S3. Relationship between the number of compounds detected in Montagu's harrier chick blood and SMI (grams). The line and shading represent the values predicted for the effect of chick SMI and the 95% confidence interval from the GLMM (Poisson distribution).

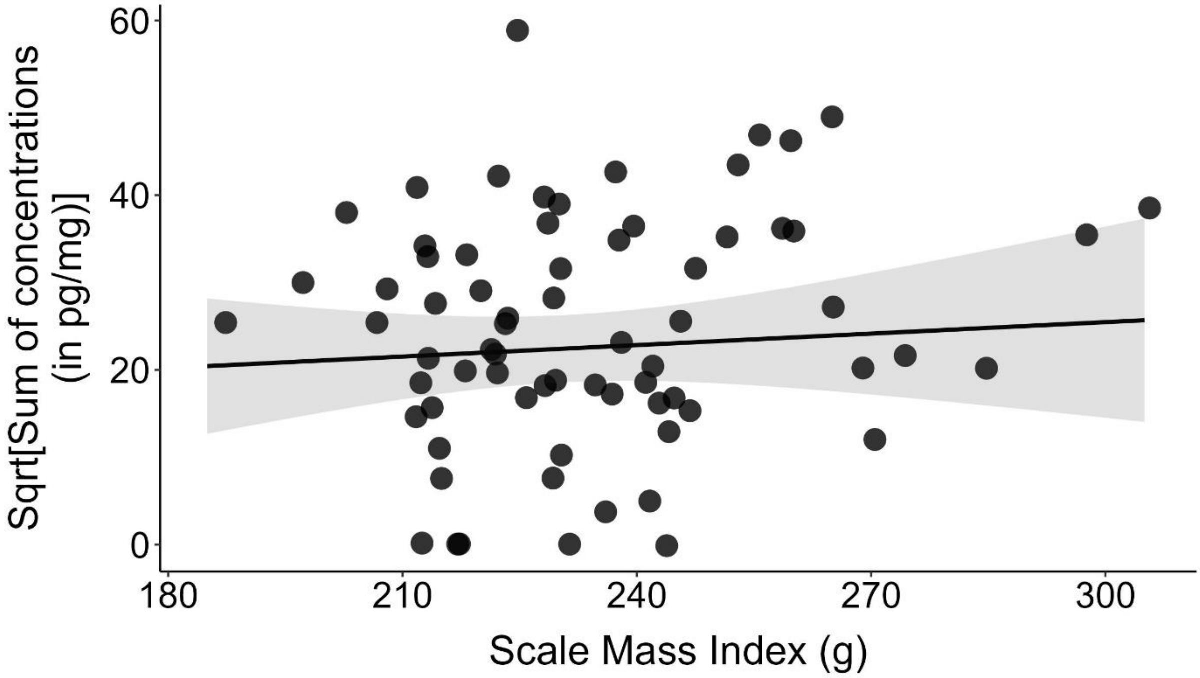


Figure S4. Relationship between the sum of pesticide concentrations (in pg.mg^{-1}, square root-transformed) detected in Montagu's harrier chick blood and SMI (grams). The line and shading represent the values predicted for the effect of chick SMI and the 95% confidence interval from the LMM.

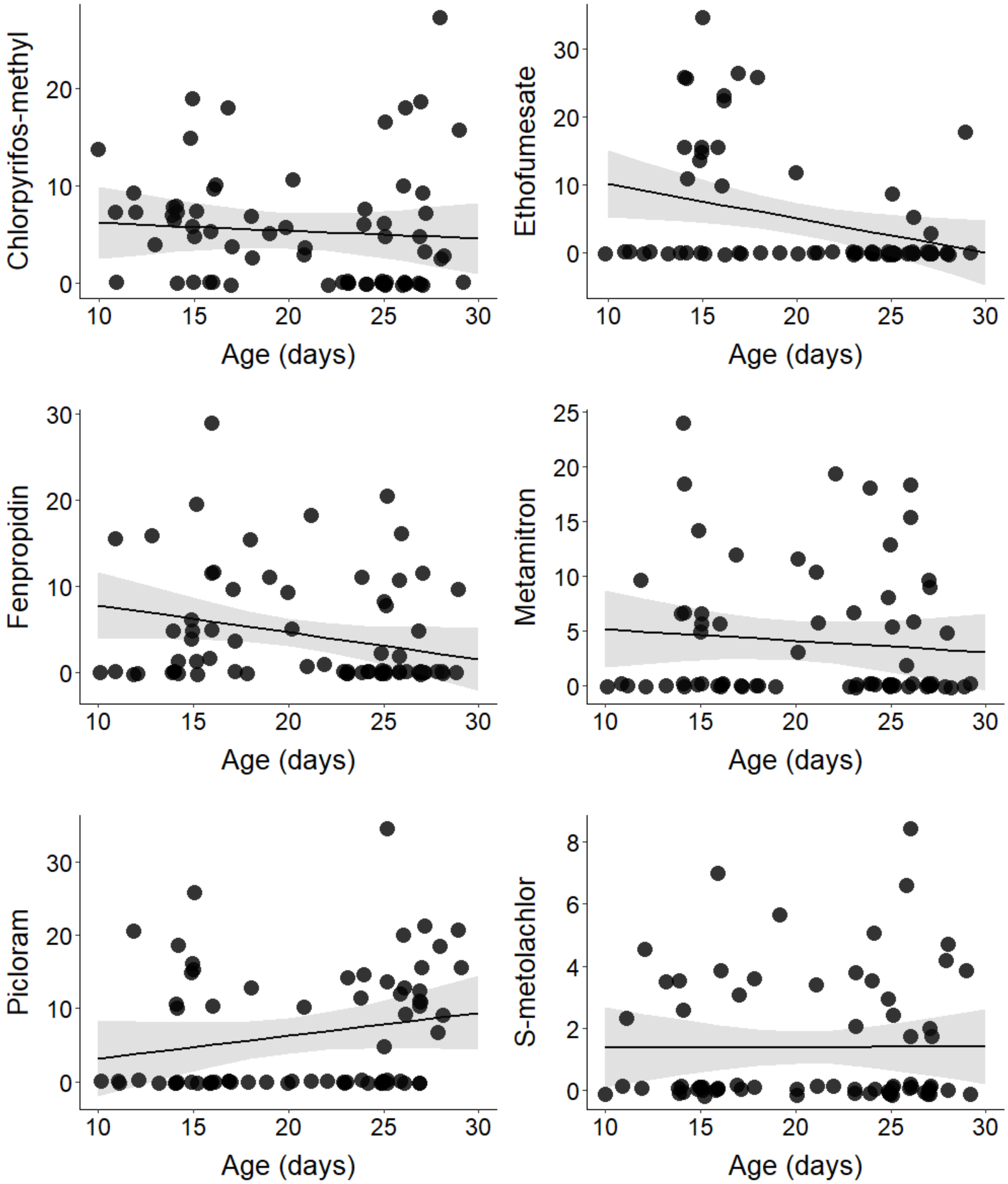


Figure S5. Changes in pesticide concentrations (pg.mg^{-1}, square root-transformed) with chick age. Lines and shading represent the values predicted for the effect of chick age and the 95% confidence interval from the LMM.

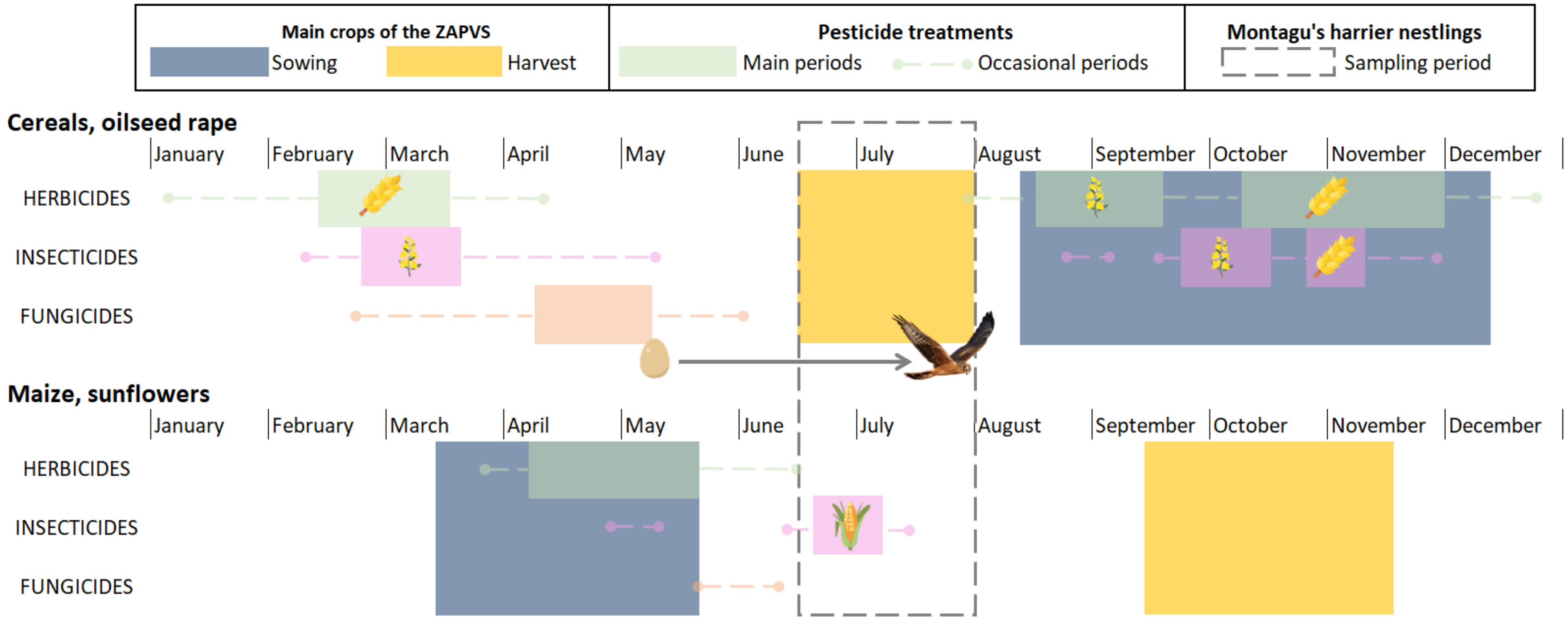
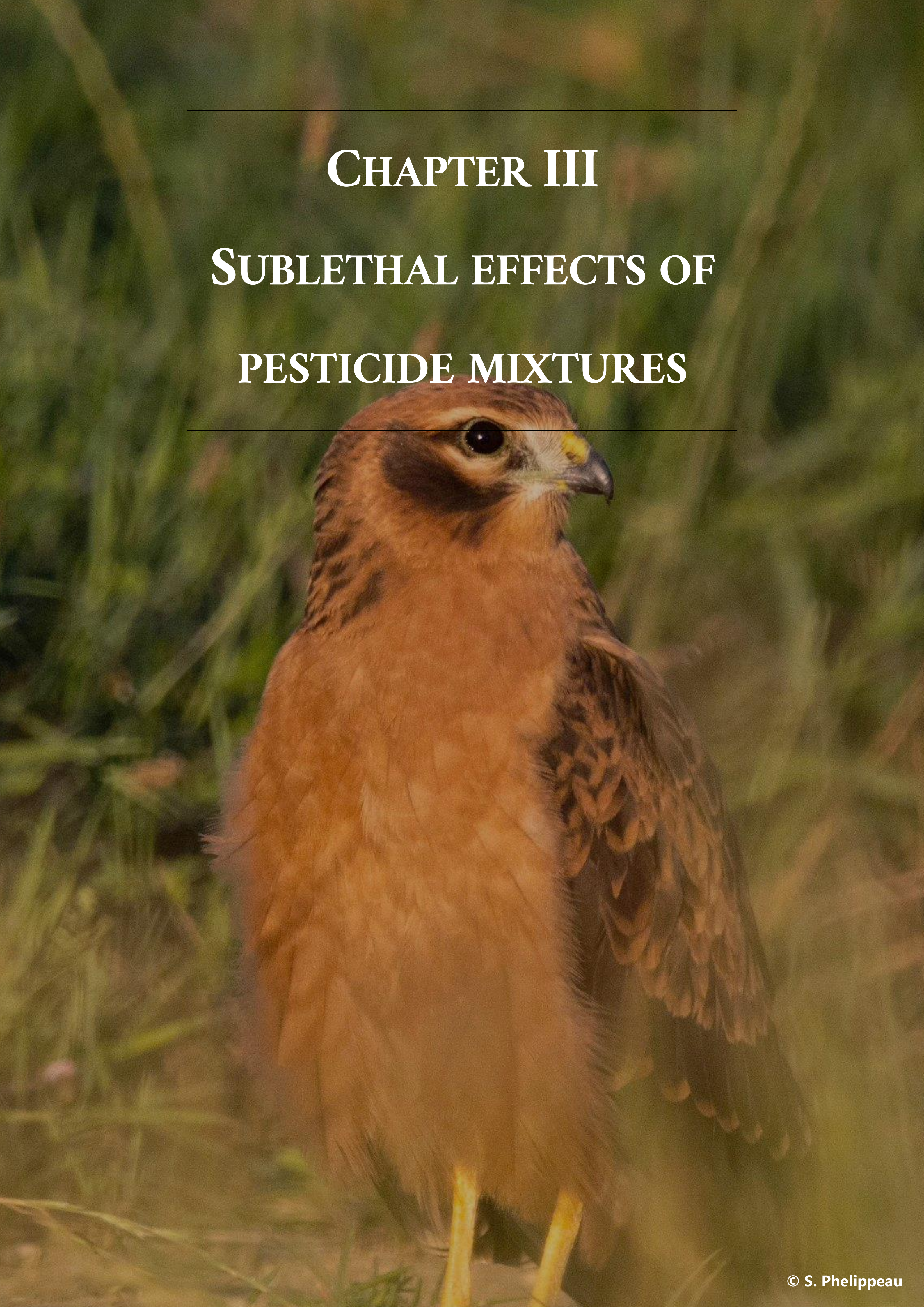


Figure S6. Theoretical calendar of the periods of pesticide application on the main crop types present in the study area. This calendar was inspired from the calendar provided by the Chamber of Agriculture of the Vienne department (available at: https://vienne.chambre-agriculture.fr/fileadmin/user_upload/Nouvelle-Aquitaine/104_Inst-Vienne/Documents/mon_exploitation/reglementation/Phytosanitaires/Calendrier_traitements_phytos_GC86.pdf) and Nièvre department (available at: https://bourgognefranchecomte.chambres-agriculture.fr/fileadmin/user_upload/Bourgogne-Franche-Comte/061_Inst-Bourgogne-Franche-Comte/CA58/Grandes_cultures/ZNT/Calendrier_traitements_GC_CA58.pdf). The timing of crop treatments depends on the growth stage of the crop, the type of phytopharmaceutical product (PPP) applied, and the weather, therefore pesticide application periods may vary locally. Spraying is the main application method although it can vary depending on the PPP and crop type. Fungicides in autumn are mainly coated around seeds prior to sowing and are thus not depicted in the calendar. When the main treatment period concerns only one of the two crops, icons are depicted inside the squares. Nestlings were sampled from mid-June until the end of July (i.e., the harvest period of the cereal crops in which the nests were located).

Chapter III

Sublethal effects of pesticide mixtures



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Summary of Chapter III

Although various studies investigated the risk pesticides pose to farmland birds, none directly assessed the contamination of wild birds with a large range of 'non-persistent' pesticides (i.e., not included in the POPs list). Besides, the sublethal effects of some substances revealed in laboratory species are hardly generalizable to wild species exposed to pesticide mixtures and facing uncontrolled conditions. Therefore, this chapter aimed to investigate the cocktail effects of pesticides on farmland birds. The monitoring of 35 nestlings during their rearing period in 2018 allowed the characterisation of their growth, physiological stress and contamination levels. In addition to the two proxies of contamination levels used in the second chapter (i.e., the number of pesticide concentrations and the total sum of their concentrations), two other metrics of contamination levels were used to avoid scarce molecules detected only in a few individuals: the number and sum of concentration of the most prevalent pesticides (detected in at least 35% of the 70 samples). The effects of nestlings' contamination levels, developmental phase (either ≤17 days old for rapid structural growth period or ≥ 18 days old) and sex on their tarsus growth, mass gain, body condition, and corticosterone secretion were investigated (*ARTICLE 5*). While none of these factors influenced the physiological stress of chicks (measured through corticosterone secretions) or their mass gain, contamination levels influenced the tarsus growth and body condition of nestlings in an age and sex-dependent manner. Males aged ≤17 days old with higher total pesticide concentrations in blood had shorter tarsus length than males with lower pesticide concentrations. Females aged ≤17 days old displayed a reversed tendency. Nonetheless, older nestlings (≥ 18 days old) displayed the exact opposite relationships and generally had expected or higher tarsus lengths. Some substances detected in heavily contaminated chicks having delayed tarsus growth, such as cypermethrin and chlorpyrifos-methyl, have been shown to cause structural abnormalities and growth retardation in birds. However, the catch-up in tarsus length later in the rearing period suggests that nestlings may compensate for the adverse effects of pesticides. Similarly for body condition, irrespective of the sex, during the phase of chicks' structural growth, nestlings with higher concentrations (among the most prevalent pesticides) displayed reduced body condition compared to nestlings with lower contamination levels, while older nestlings displayed a reversed relationship. This may be the consequence of reduced access to food items of heavily contaminated young nestlings due to the neurotoxic effects of some substances altering their behaviour. Moreover, this can result from reduced nutrient absorption due to the alterations of hormonal secretions, metabolic pathways and gut microbiome by some pesticides. Despite the absence of a general trend, these results provide the first evidence *in natura* of the adverse effects of pesticide mixtures and are in line with previous studies assessing pesticide sublethal effects in birds.

ARTICLE 5

PESTICIDE MIXTURE EFFECTS ON PHYSIOLOGICAL STRESS AND MORPHOLOGY OF GROWING WILD NESTLINGS

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In preparation for *Journal of Hazardous Materials*

Abstract

Synthetic pesticides, increasingly used in conventional agriculture, may pose a risk to non-target organisms notably farmland birds, through their hazardous effects on life-history traits. However, the cocktail effects of pesticides on chicks' growth and physiological stress have never been assessed *in natura*. We aimed to investigate the effects of pesticide contamination levels on tarsus growth, mass gain and body condition of 35 nestlings of *Circus pygargus*, a farmland apex predator, blood-sampled twice during their development. We also investigated the effects of pesticide contamination levels on these nestlings' physiological stress through the assessment of their basal and stress-induced corticosterone secretion. We found contamination levels ranging from 0 to 9 pesticides detected and up to 3472.68 pg.mg^{-1} of summed concentrations. We did not observe a general pattern of pesticide mixture effects on nestlings' growth but rather an age and sex-dependent altered growth. Males under 17 days old with higher total sum of pesticide concentrations had shorter tarsus than nestlings with lower pesticide load. Similarly, the body condition of nestlings aged under 17 days old was poorer when they had higher summed concentrations of the most prevalent pesticides, irrespective of their sex. However, we did not find a relationship between contamination levels and any of the corticosterone secretion, suggesting that this metric of physiological stress may be less sensitive to pesticide effects. Besides, the delayed growth (tarsus and body condition) observed in young nestlings is caught up later during their development. Therefore, our study did not find a clear effect of pesticide mixtures on the development of wild Montagu's harrier nestlings. Including other life-history traits would provide more information on other stress endpoints and the potential trade-offs in energy investment between an active defence against pesticides and important functions of the organism.

Keywords: body condition, corticosterone, mass, Montagu's harrier, multiresidue analysis, raptor, tarsus

1. Introduction

Synthetic pesticides and fertilizers used in conventional agriculture have been pointed out as the main factors driving farmland birds' population decline (Rigal et al., 2023). Although the toxicity to non-target birds of legacy pesticides – either Persistent Organic Pollutants (POPs) or not – has been highlighted in various field and experimental studies (see Mitra et al., 2011; Moreau et al., 2022 for reviews), the toxicity of currently used pesticides, supposed to be of low-risk, is still under debate (Carpy et al., 2000; Storck et al., 2017; Rezende et al., 2021). Yet, non-persistent pesticides may have both indirect effects on birds through the reduction of resources (Gibbons et al., 2014) and direct effects through lethal or sublethal effects (i.e., affecting the organism in the long term; Moreau et al., 2022). Besides, most studies considering non-persistent pesticide effects on birds usually investigated the effects of a single class of pesticides under experimental conditions (but see mixtures of different classes on *Apis mellifera*, Almasri et al., 2020; triazole fungicides mixtures on *Alectoris rufa*, Fernández-Vizcaíno et al., 2024), neglecting the effects of complex mixtures of more than one pesticide class *in natura*. Thus, there is a need to investigate the underlying mechanisms linking pesticide contamination levels to farmland birds' decline under field conditions (Moreau et al., 2022).

The developmental period is a critical period for organisms, being highly demanding in energy and implying many endocrine systems for its regulation (McNabb, 2007; Wada, 2008). Glucocorticoids for instance are stress-associated hormones that interact with thyroid hormones involved in the development of chicks; an altered secretion might thus affect the maturation of different organs and growth (McNabb, 2007; Wada & Breuner, 2008; Schoech et al., 2011). Among glucocorticoids, corticosterone is a widely used physiological endpoint for bird species, interesting for measuring the impact of both short-term and long-term stressors (i.e., lasting minutes to months, Gormally & Romero, 2020). A chronic increase in corticosterone levels may result in fat store depletion, muscle loss, growth and immune system suppression notably (Baos & Blas, 2008). Besides its impacts on chicks' growth, corticosterone levels may modulate the behaviour, as high levels would increase aggressiveness and begging behaviours in nestlings which may be important in sibling competition for food (Kitaysky et al., 2003). Although it is acknowledged that this mechanism allows hierarchies among siblings to be maintained (Love et al., 2003a), high corticosterone levels in juveniles might result in long-term consequences later in adulthood, notably on personality (Schoech et al., 2011). Some environmental pollutants might disrupt an individual's homeostasis by acting as endocrine disruptors which may further result in altered growth (Giesy et al., 2003; Baos & Blas, 2008). In fact, depending on the contaminant, exposure of nestlings may either increase or inhibit their stress responses (Baos et al., 2006; Franceschini et al., 2008, 2009). Moreover, the detoxification of pollutants might increase energetic costs leading to elevated corticosterone secretion and draining resources from the limited energy pool available for nestling growth (Monclús et al., 2019). Therefore, exposure to contaminants during nestling development might slow down their growth and even increase their mortality (Spanh & Sherry, 1999). For instance, barn owls (*Tyto alba javanica*) nestlings fed with prey contaminated with rodenticides were smaller than nestlings fed with uncontaminated prey (Naim et al., 2010). Similarly, offsprings of grey partridges (*Perdix perdix*) fed with conventional grains (i.e., with a mixture of pesticide residues) were smaller at hatching than offsprings from breeding pairs fed with organic grains (Gaffard et al., 2022). However, at 24-days old they caught up size but their body mass index was lower, suggesting that parents' exposure to pesticides might have affected chicks' mass gain but not structural growth (Gaffard et al., 2022). In red-legged partridges (*Alectoris rufa*), chicks from parents fed with thiram-treated grains displayed slower growth rates than control chicks (Lopez-Antia et al., 2015a) while growth rates of chicks were unaffected when parents were fed with grains coated with a mixture (imidacloprid, thiram and difenoconazole; Lopez-Antia et al., 2013) or with imidacloprid alone (Lopez-Antia et al., 2015b). Therefore, it appears that the effects of indirect nestling contamination (i.e., through parental exposure) on their growth vary depending on the pollutants concerned. Still, a shared shortcoming in all these studies is that chicks were not sampled, although this would have been useful to directly associate their pesticide contamination levels to consequences on their growth. Nonetheless, there are some studies directly assessing contaminants in nestlings. For instance, high levels of POPs in the liver of European shag (*Phalacrocorax aristotelis*) chicks result in strong fluctuating asymmetry of wing bone length indicating that these compounds might have affected nestlings' growth although these were not related to endocrine disruptions (Jenssen et al., 2010). Moreover, Bonelli's eagle (*Aquila fasciata*) chicks with high levels of DDT in their blood had reduced body condition (Ortiz-Santaliestra et al., 2015), and bromadiolone detection in the blood of fledglings of common kestrel (*Falco tinunculus*) was associated with lower body mass (Martínez-Padilla et al. 2017). However, there is a lack of knowledge concerning the effects of pesticide mixtures (other than POPs) on wild nestling growth and hormone secretion even though currently used pesticides are suspected to have endocrine-disrupting properties (McKinlay et al., 2007).

Farmland birds such as the Montagu's harrier (*Circus pygargus*) are exposed to pesticide mixtures all along their lives. As for other ground-nesting species, hatchlings might be contaminated from the direct exposure of eggs to pesticides (e.g., grey partridges, Bro et al., 2016 or red-legged partridges Ortiz-Santaliestra et al., 2020) and maternal transfer of compounds from females to their eggs (e.g., Bellot et al., 2022). This basal contamination level of hatchlings reduces with chicks' age and is lower at fledging (Fuentes et al., 2024a). Nonetheless, nestlings are exposed to pesticides all along the rearing period through contact (e.g., with the soil, vegetation, dust, rain and runoff), inhalation of volatile substances that drifted, and consumption of contaminated prey (Fuentes et al., 2024b). Moreover, Montagu's harrier displays hatching asynchrony (1 to 2 days between hatching events) and sexual dimorphism, with females being heavier than males (~19% visible during the second part of the rearing period; Millon & Bretagnolle, 2005), leading to hierarchies among nestlings. First-hatched chicks and females are generally advantaged in sibling competition for food within a brood (Arroyo, 2002), although the sex of chicks does not seem to influence their corticosterone production (Rabdeau et al., 2019). Montagu's harrier nestlings are thus particularly interesting to study the sublethal effects of pesticides on their growth and stress-associated hormones.

In this study, we sought to investigate the effects of pesticide contamination levels on both the physiological stress of Montagu's harrier chicks and their growth. We screened 116 compounds in chicks blood-sampled twice during their growth, characterizing their contamination levels through 4 metrics: total number of pesticides detected, total sum of pesticide concentrations, number of pesticides detected among the most prevalent (present in more than 35% of samples), and the sum of concentrations among the most prevalent pesticides. Although expectations concerning chicks' physiological stress are difficult to raise because pesticide mixtures may either increase or decrease hormone production, we expected high contamination levels to induce increased corticosterone secretions, altered body condition and delayed growth (skeletal growth and mass gain). However, predictions on growth might be reversed due to its influence on contaminant deposition rates, leading to a stronger dilution effect in faster-growing chicks (Goutner et al., 2001).

2. Materials and Methods

2.1. Ethical statement

The handling of Montagu's harrier chicks was allowed and licensed by the Centre de Recherches sur la Biologie des Populations d'Oiseaux (CRBPO; Museum National d'Histoire Naturelle; licence #1308) and all experiments followed the French guidelines for the ethical use of animals in research (APAFIS#18557–2019010822312199v2).

2.2. Study area and model species

2.2.1. Study area

The study area is located in southwestern France (46°11'N, 0°28'W), in the Zone Atelier Plaine & Val de Sèvre (ZAPVS), a Long-Term Social-Ecological Research Platform (LTSER) of 450 km^2. The area is predominantly covered by intensively managed cereal crops (~41% of the cultivated area between 2009 and 2016; Bretagnolle et al., 2018). Organic farming crops (i.e., not using synthetic pesticides and fertilizers; Regulation EU, 2018/848) accounted for ~11% of the ZAPVS surface in 2018 (Fig. 1).

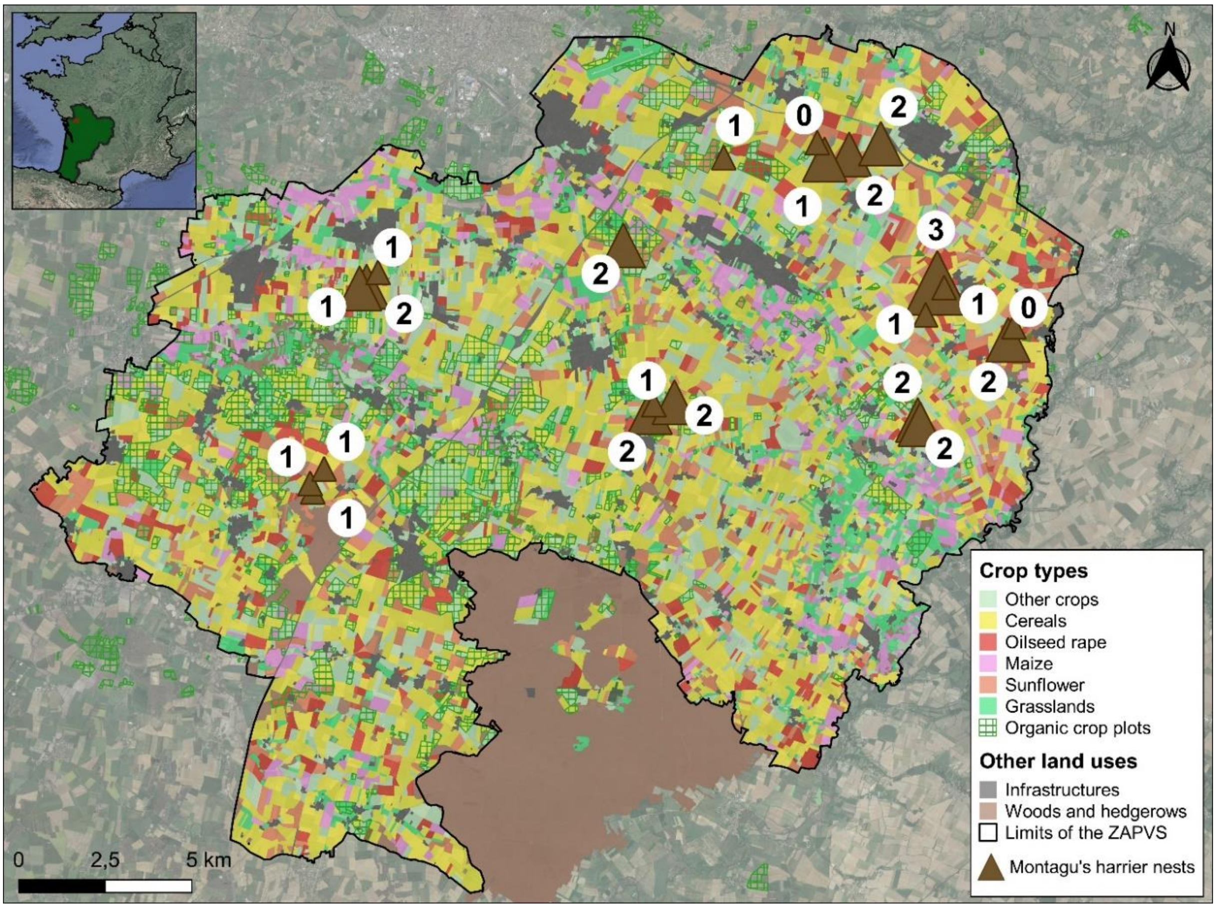

Figure 1. Localization of the 22 nests of Montagu's harrier (*Circus pygargus*) monitored in 2018 in the Zone Atelier Plaine & Val de Sèvres (ZAPVS). The size of the circles reflects the number of siblings from the brood sampled for pesticide analyses (up to 3 chicks). Number in circles indicates the number of siblings with a complete corticosterone dataset available. The organic crop plots represented correspond to the year 2018.

2.2.2. Model species

The Montagu's harrier has been monitored in the ZAPVS since 1994 (Bretagnolle et al, 2018). The individuals of the population studied in this area overwinter in western Africa and nest on the ground of cereal crop plots, laying up to five eggs (exceptionally eight; Arroyo et al., 1998; García & Arroyo, 1998). The incubation lasts 29 days and the rearing period 30 to 35 days (Arroyo et al., 2007). Montagu's harrier breeding success depends mainly on the availability of its main prey, the common vole (*Microtus arvalis*) although the species also preys upon orthopterans and passerine birds (Salamolard et al., 2000). The breeding success in this site is ~2.05 fledglings per breeding attempt (Arroyo et al., 2004), with brood sizes varying between one and five nestlings (see Fuentes et al., 2023 for an overview of several years). Males ensure food provisioning of incubating females and chicks, hunting within home ranges of ~14 km^2 in the study area (Salamolard, 1997). Females contribute to the food provisioning of chicks by the end of the rearing period, hunting close to the nest (García & Arroyo, 2005).

2.3. Data collection

From mid-April 2018, Montagu's harrier nests were searched, located and recorded using coordinates on a geographical information system (GIS; QUANTUMGIS 3.22.16; QGIS Development Team, 2023; Fig. 1). Nests were visited twice before hatching and every week afterwards (lag time of 7 ± 2 days between visits; Fig. 2). Chicks were thus monitored during 4 visits at least (except for nests discovered after hatching). A total of 81 chicks (24 nests) were monitored between early June and early August 2018, although only 35 of them (22 nests) were included in the present study due to the absence of samples for the rest of the nestlings (see section 2.3.3.1. Blood sampling). No sex-ratio bias was observed that year (36 females and 45 males; Binomial test $p = 0.37$), even considering only the 35 chicks included in the present study (17 females and 18 males; Binomial test $p = 1.00$).

Figure 2. Montagu's harrier (*Circus pygargus*) nestlings at different developmental stages: (a) four chicks of 4-6 days old, (b) four chicks of 11-17 days old, (c) three chicks of 18-23 days old, and (d) four chicks of 22-27 days old. Photo credit: E. Fuentes.

2.3.1. Experimental design

During the hatching visit, chicks were head-marked using water-based coloured pens, allowing their individual identification (Fig. 2a). Morphometric measures of chicks were taken during all nest visits and included tarsus length, wing length and body weight (see section 2.3.2). At the second (16 ± 3 days-old chicks Fig. 2b) and fourth visit (26 ± 2 days-old chicks, Fig. 2d) of the nests, a specific protocol was followed (Fig. 3). When arriving at nest, chicks were caught and carefully handled to collect a first blood sample (see section 2.3.3) in the shortest time possible (on average at 2min 20s ± standard deviation 45 s), then morphometric measures were taken (see section 2.3.2). The handling time was minimised to reduce potential stress and a second blood sample was collected (at 21min 23s ± 7 min 39s) before chicks were released at their nest. During the second visit, chicks were also banded with an aluminium ring engraved with a unique code and provided by the Museum National d'Histoire Naturelle de Paris (France), and sexed according to the colour of their iris (brown for females and grey for males; Leroux & Bretagnolle, 1996). The age of nestlings was estimated *a posteriori*, using a morphometric estimation of age (function using wing length measure [Arroyo, 1995]; calculator available at https://busards.com/index.php/Outils/biometry).

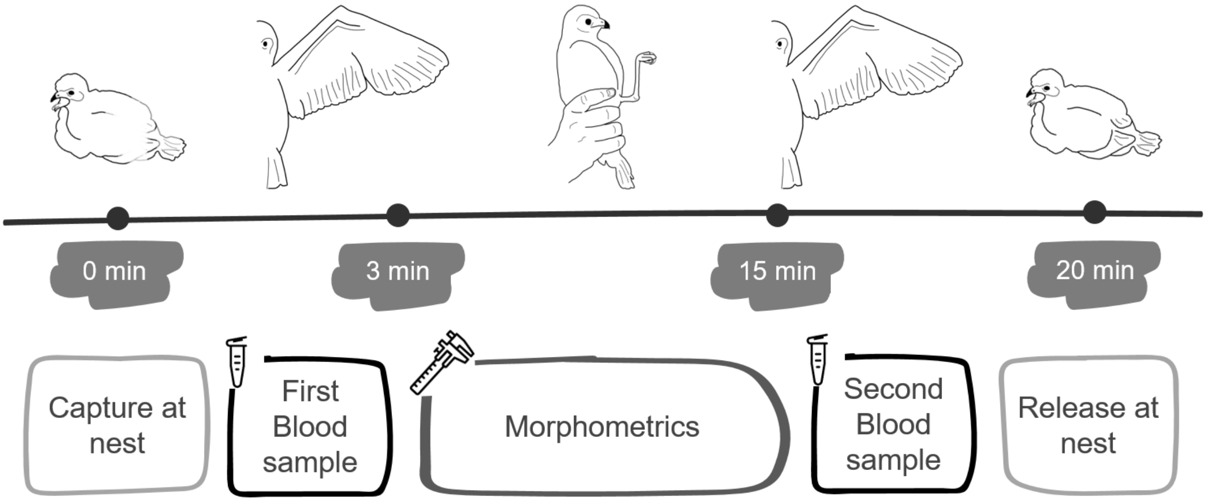

Figure 3. Procedure followed during the second and fourth visits at Montagu's harrier (*Circus pygargus*) nests, when chicks were ~16 and ~26 days-old respectively.

2.3.2. Morphometric measures

The mass of hatched chicks was obtained by weighing them with a Pesola 500 g digital scale (accuracy ± 0.1 g), then after the first week, they were weighed with a Pesola 500 g spring scale (accuracy ± 5 g). Tarsus length was measured with a digital calliper (accuracy ± 0.1 mm) and wing length with a ruler (accuracy ± 1 mm). Generally, the right limbs of chicks were measured twice during the first weeks and the left limbs were measured twice only for the last visit. Consequently, the mean tarsus length was used in further analyses.

2.3.2.1. Growth parameters

Growth parameters aimed to reflect the delayed or advanced growth of chicks compared to theoretical growth curves. In Montagu's harrier, tarsus length and body mass increase with age following an asymptotic curve, therefore, theoretical growth curves were obtained by fitting a Gompertz model (Millon et al., 2008) to all tarsus length or mass measures taken during nestlings' growth (i.e., from the first post-hatching visit to the last one) (see Figure S1 and S2 in supplementary materials). Then, we extracted the residuals of the non-linear regression (Gompertz model; Millon et al., 2008) of tarsus length on age (skeletal growth) and of weight on age (mass gain). The species being sexually dimorphic, the sexes were separated to obtain sex-specific growth curves and extract residuals of both metrics separately (Figure S1, S2). These residuals were then used as response variables in statistical analyses (see section 2.4).

2.3.2.2. Body condition

The body condition of chicks was estimated using the scale masse index (SMI) following Peig & Green (2009), calculated for males and females separately. This index is supposed to reflect the relative size of energy reserves, contrary to raw mass that can be influenced by recently ingested food. The body condition of chicks tends to decrease with age because of the reduction of body fat to attain the optimum mass for flight (Mauck and Ricklefs, 2005; Wright et al., 2006), thus we extracted the residuals of the linear regression of SMI on age (one for each sex; see Figure S3 in supplementary materials) to reflect the excess or deficit of lipid stores of chicks. These residuals were then used in statistical analyses (see section 2.4).

2.3.3. Blood collection and analyses

2.3.3.1. Blood sampling

Each blood sampling consisted of a puncture in the brachial vein using a sterile needle and heparinized capillaries to collect blood. For the first blood sample, one Eppendorf tube containing ~150 µL was stored in a cooler (0-5°C) until returning to the laboratory for baseline corticosterone analyses (see section 2.3.2.2). The second blood sample was collected into two Eppendorf tubes, one for pesticide and one for stress-induced corticosterone analyses containing ~50 µL and ~150 µL of blood respectively. The tubes were also stored in the cooler until further analyses in the laboratory. Blood samples for pesticide titration were available for 35 chicks at both the second and fourth visits. Among these chicks, some had no blood sample available for corticosterone titration, either for an entire visit (2 nestlings) or the second blood sample of a visit (3 nestlings). Thus, 30 chicks had a complete set of corticosterone data available (two samples at two visits, i.e., four samples in total).

2.3.3.2. Corticosterone titration

Both blood samples of ~150 µL were centrifuged 10 min at 9000-rpm (Bio Lion XC-LED12K) to collect the plasma and stored at -20°C until corticosterone titration. Following Lormée et al. (2003), from 30 µL of plasma, corticosterone was extracted using ethyl ether and titrated by radioimmunoassay. Three concentrations of corticosterone (ng.µL^{-1}) per chick per visit were obtained: the baseline corticosterone (BASECORT), the maximal corticosterone (MAXCORT) and the stress-induced response in corticosterone (REPCORT which is the MAXCORT minus the BASECORT). The BASECORT and REPCORT were not correlated to each other (Pearson's correlation test, $\rho = -0.002$, 95% CI = [-0.24; 0.25]). Although the MAXCORT was correlated to both BASECORT ($\rho = 0.36$, 95% CI = [0.09; 0.58]) and REPCORT ($\rho = 0.93$, 95% CI = [0.90; 0.96]), we kept this measure because it represents the maximum level of corticosterone experienced by nestlings during handling while REPCORT reflects individual plasticity in their stress-induced corticosterone secretion, and because it is not dependent on the timing of sampling contrary to BASECORT. To stay consistent in our methodology and because both baseline and stress-induced corticosterone concentrations increase with age due to the development of the HPA axis (Love et al., 2003b; Wada, 2008), we extracted the residuals of the linear regression of BASECORT, REPCORT and MAXCORT on chicks' age for each sex separately (see Figure S4 in supplementary materials), reflecting the excess or deficit of hormone secretion of chicks. Residuals were then used as response variables in models implemented for statistical analyses (see section 2.4).

2.3.3.3. Pesticide titration

Following Rodrigues et al. (2023), 50 µL of whole blood (i.e., red blood cells and plasma) were used for multiresidue analyses. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and automated thermal desorption gas chromatography coupled to tandem mass spectrometry (ATD-GC-MS/MS) were performed to detect and quantify 116 compounds in MRM detection mode for both instrumentations. These 116 compounds include herbicides, fungicides, insecticides, a safener (improving herbicide selectivity towards weeds rather than crop plants) and a synergist (increasing the action of pesticide), among the most used in France. Twelve compounds were added to the initial 104 compounds validated by Rodrigues et al. (2023) after their optimization and validation. The limits of detection (LODs) and limits of quantification (LOQs) varied between 0.001 and 0.124 $pg.mg^{-1}$ and between 0.004 and 0.436 $pg.mg^{-1}$ respectively. The LODs and LOQs for each compound detected in the present study are provided in supplementary material (Table S1). For further details concerning instrumentations, calibrations, analytical quality assurance and control procedures, refer to Rodrigues et al. (2023).

2.4. Statistical analyses

For each chick, contamination levels were estimated by four metrics: (1) the total number of compounds detected in blood (N_{tot}), and (2) the total sum of compound concentrations in blood ($\sum tot$). Because the total number of molecules or concentrations include a strong variation among individuals, keeping only the most prevalent molecules would avoid the "noise" from keeping scarce molecules. Therefore, (3) the number of compounds detected among the most prevalent (N_{preval}) and (4) the sum of concentrations among the most prevalent compounds ($\sum preval$) were also used as contamination metrics. The most prevalent molecules were defined as those detected in at least 35% of the samples (i.e., at least detected in 25 of the 70 samples; see Table 1), this threshold being the third quartile prevalence among the 18 molecules (i.e., the 25% molecules with the highest prevalence). Consequently, the number and sum of concentrations of the most prevalent molecules included chlorpyrifos-methyl, fenpropidin, metamitron, metolachlor-S, and picloram. Although the four metrics moderately to strongly correlate to each other (Pearson's correlation test ρ between 0.38 and 0.82, see Figure S5 supplementary materials), we decided to keep all of them as we do not expect the same influence on the studied traits. Indeed, the presence of compounds (i.e., the number) might have different effects depending on their relative quantity (i.e., the concentrations). For ∑tot and ∑preval, values were square root transformed before analyses to ensure a normal distribution and allow better visualization.

The residuals of growth parameters (tarsus and mass), body condition, BASECORT, REPCORT and MAXCORT, were used as response variables in Linear Mixed Effect Models (LMMs). One model per metric of contamination level was implemented, thus four LMMs per trait were set. Because chicks were sampled twice and may belong to the same nest, we used the identity of chicks nested within nest identity as random effects in all models.

The LMMs using tarsus, mass and body condition residuals as response variables included the contamination level, sex and age in three-interaction fixed effects. For the effect of age, two classes were defined: the first corresponding to ages of 10 to 17 days and the second corresponding to ages of 18 to 29 days. These classes were defined according the staggered developmental pattern of semi-altricial nestlings that display a rapid structural growth during the first half of the rearing period (Fig. 2a, 2b) and then a level off by the second period that coincides with the period of rapid growth in feathers (Fig. 2c, 2d) (Jongsomjit et al., 2007; but see Koga et al., 1989; Arroyo et al., 2000; Choi et al., 2015 for examples on other raptor species). The rearing period of Montagu's harrier nestlings lasting up to 35 days, expectations on the effects of contamination levels on chicks' growth before 17 days old might differ from expectations afterwards when structural growth slows down (see supplementary Fig. S1 and S2).

For the LMMs using BASECORT, REPCORT and MAXCORT residuals as response variables, the contamination level, sex and age were again included as three-interaction fixed effects, although the age was left as a continuous numeric variable (the change in corticosterone secretion with age is not staggered as growth components). To these effects were added the brood size and hatching order as fixed effects because these factors may influence corticosterone levels in birds (Love et al., 2003a).

All models were implemented using the package 'lme4' (Bates et al., 2015) and models residuals were checked using the package 'DHARMa' (Hartig, 2022) in R v.4.2.2 software (R Core Team, 2022). We tested the significance of all the explanatory variables through a type III analysis of deviance using the likelihood ratio-based χ^2-statistics from the package 'car' (Fox & Weisberg, 2011) and provided the results in supplementary materials. When interacting effects were statistically supported, plots were represented using the interact_plot function of the package 'interactions' (Long, 2019).

3. Results

3.1. Pesticide contamination levels

Among the two sampling occasions of the 35 chicks, 18 pesticides had concentrations > LOD (9 herbicides, 5 insecticides and 4 fungicides; see Table 1). Considering all samples, the average number of pesticides detected in total (N_{tot}) or among the most prevalent molecules (N_{preval}), and the total concentrations (∑tot) or among the most prevalent (∑preval) in chicks' blood, are reported in Table 2 with their associated minimum and maximum values. More details concerning the number and concentrations of pesticides at each sampling occasion can be found in Fuentes et al. (2024a).

Table 1. Pesticides detected in the blood of 35 Montagu's harrier (*Circus pygargus*) chicks in 2018. Bold italic compound names and total prevalence indicate the most prevalent molecules included in the metrics of contamination levels (number and sum of concentrations).

Molecule	*Type* / Family	Mode of action	Ban in France	Prevalence (in %)		
				Total	First samples	Second samples
Acetamiprid	*Insecticide* / Neonicotinoid	Nicotinic acetylcholine receptor (nAChR) competitive modulators	2018	15.7	-	31.4
Aclonifen	*Herbicide* / Diphenylether	Inhibition of solanesyl diphosphate synthase (SDPS)		8.6	17.1	-
Bupirimate	*Fungicide* / Pyrimidine	Nucleic acid metabolism (adenosine-deaminase)		15.7	31.4	-
Carbaryl	*Insecticide* / Carbamate	Acetylcholinesterase (AChE) inhibitors	2007	25.7	51.4	-
Chlorpyrifos-methyl	*Insecticide* / Organophosphate	Acetylcholinesterase (AChE) inhibitors	2020	***61.4***	77.1	45.7
Cypermethrin	*Insecticide* / Pyrethroid	Sodium channel modulators		32.9	65.7	-
Dicamba	*Herbicide* / Benzoates	Auxin mimics		24.3	48.6	-
Dichlobenil	*Herbicide* / Nitrile	Inhibition of cellulose synthesis	2008	5.7	11.4	-
Ethofumesate	*Herbicide* / Benzofuran	Inhibition of very long-chain fatty acid synthesis (VLCFA)		27.1	42.9	11.4

Fenpropidin	*Fungicide /* Piperidine	Inhibition of sterol biosynthesis in membranes		***48.6***	65.7	31.4
Fenpropimorph	*Fungicide /* Morpholine	Inhibition of sterol biosynthesis in membranes	2019	20.0	-	40.0
Metamitron	*Herbicide /* Triazinone	Inhibition of photosynthesis at photosystem II		***40.0***	45.7	34.3
Metolachlor-S	*Herbicide /* Chloroacetamide	Inhibition of very long-chain fatty acid synthesis (VLCFA)	2024	***35.7***	34.3	37.1
Penconazole	*Fungicide /* Triazole	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))		17.1	-	34.3
Picloram	*Herbicide /* 6-Chloropicolinates	Auxin mimics		***45.7***	34.3	57.1
Propazine	*Herbicide /* Triazine	Inhibition of photosynthesis at photosystem II	2002	14.3	-	28.6
Prosulfocarb	*Herbicide /* Thiocarbamate	Inhibition of very long-chain fatty acid synthesis (VLCFA)		17.1	34.3	-
Thiamethoxam	*Insecticide /* Neonicotinoid	Nicotinic acetylcholine receptor (nAChR) competitive modulators	2018	1.4	-	2.9

Table 2. Descriptive statistics of the four metrics of contamination levels determined in Montagu's harrier nestlings. Mean and standard deviation (SD) were obtained from the samples among the 70 analyses that had a detection (i.e., above the LOD threshold).

Metrics of contamination	**Mean ± SD**	**Minimum**	**Maximum**
N_{tot}	4.57 ± 2.33	0	9
N_{preval}	2.31 ± 1.25	0	5
∑tot (in pg.mg^{-1})	780.48 ± 693.73	<LOD	3472.68
∑preval (in pg.mg^{-1})	297.86 ± 294.67	<LOD	1223.11

3.2. Influence of contamination levels on chicks growth and physiological stress

As all analyses were performed on the residuals extracted from growth curves or linear temporal changes, the terms larger or smaller (or any comparative) used hereafter refer to the expected values (in tarsus length, BASECORT, etc.) of chicks during their development. Negative residuals indicated a deficit or delayed status compared to the reference state obtained from the studied chick population, and positive residuals an excess or advanced status.

3.2.1. Skeletal growth

Only the total sum of compound concentrations in blood (∑tot) in interaction with chicks' sex and age significantly influenced chicks' tarsus length (Table S4). During the structural growth period (10-17 days), males with higher ∑tot had shorter tarsus (i.e., negative residuals) than low-contaminated males (Fig. 4). Females had a reversed relationship, although they mainly keep a deficit in tarsus length whatever their pesticide concentrations (Fig. 4). These patterns were reversed in the second phase of the rearing period (18-29 days), males tarsus tended to be larger in more contaminated chicks, while females had a decreasing trend (i.e., larger tarsus length at low pesticide concentrations and smaller-to-expected tarsus length at high pesticide load) (Fig. 4).

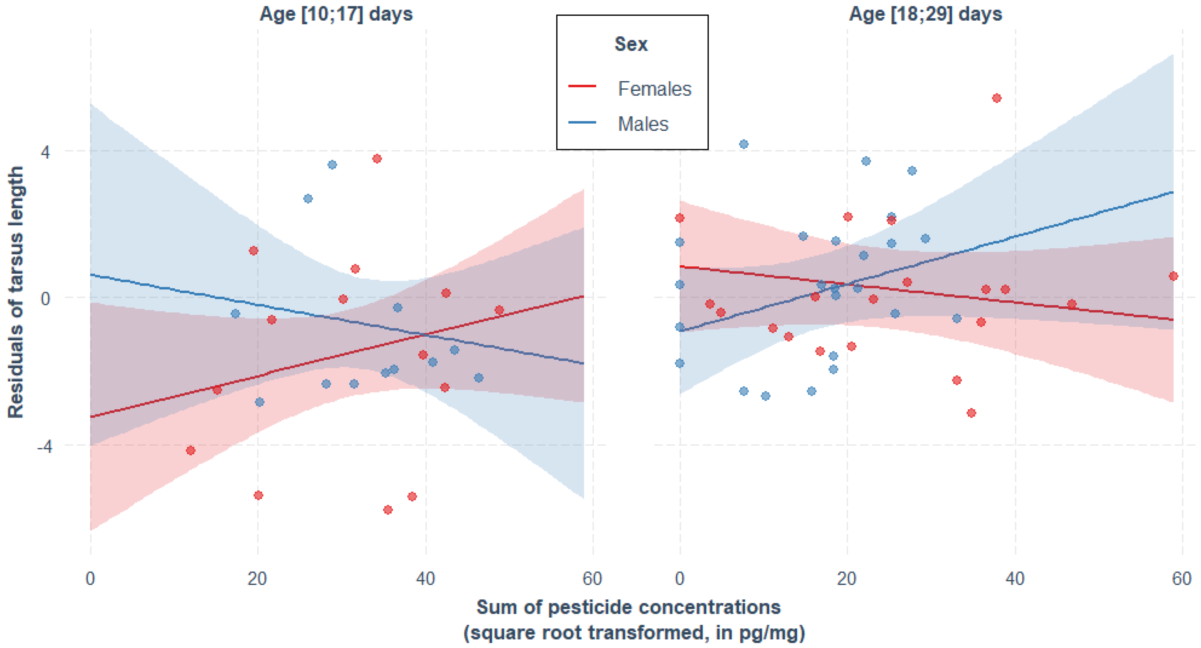

Figure 4. Effect of the triple interaction of pesticide contamination (sum of pesticides concentrations) and nestlings' sex and age on their residual tarsus length. Chicks sampled during the structural growth period (10-17 days) are represented on the left and during the second part of the rearing period (18-29 days) on the right. The regression lines represent the predicted values for the effect of age on the residuals of tarsus length from the LMM and the shadings represent the associated 95% confidence intervals. The colour of points, regression lines and shadings correspond to females in red and males in blue.

3.2.2. Mass gain

None of the pesticide contamination metrics significantly influenced the mass residuals of Montagu's harrier nestlings, just as sex and age which did not affect mass residuals (Tables S2, S3, S4 and S5 in supplementary materials).

3.2.3. Body condition

No contamination level metrics in interaction with sex and age significantly affected chicks' body condition (Table S2, S3, S4 and S5). However, the sum of concentrations of the most prevalent compounds (∑preval) in interaction with nestlings' age influenced the residuals of body condition (measured through the SMI) (Table S5). Regarding the most prevalent pesticides, the more contaminated were nestlings, the larger the deficit in body condition, whatever their sex (Fig. 5). This relationship found during the first part of the growth period (10-17 days) was not found in older nestlings (18-29 days old) and slightly reversed with some chicks having high pesticide load displaying an excess in body condition (Fig. 5).

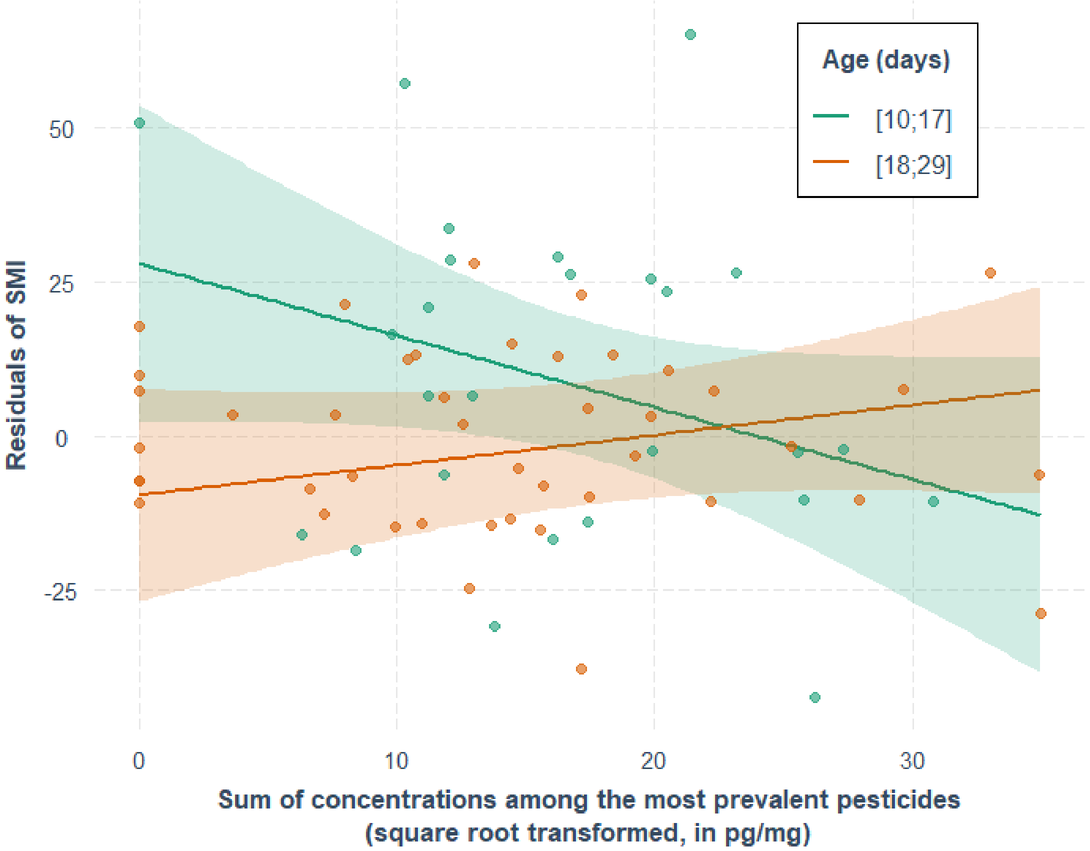

Figure 5. Effect of the double interaction of pesticide contamination (sum of concentrations among the most prevalent pesticides) and nestlings' age on their residual body condition (measured through the Scale Mass Index, SMI). The regression lines represent the predicted values for the effect of pesticide contamination on the residuals of SMI from the LMM and the shadings represent the associated 95% confidence intervals. The colour of points, regression lines and shadings correspond to chicks sampled in the structural growth period (10-17 days) in green and during the second part of the rearing period (18-29 days) in orange.

3.2.4. Corticosterone

There was no effect of the contamination levels, chicks' age and sex, brood size or hatching order on either BASECORT, REPCORT or MAXCORT residuals (Table S2, S3, S4 and S5 in supplementary materials).

4. Discussion

In the present study, no general pattern of pesticide effects on wild nestlings' development was found. Nonetheless, we detected a signal of altered growth in wild nestlings due to their contamination with pesticide mixtures. The skeletal growth of chicks can be affected by the total sum of pesticide concentrations in blood during the phase of rapid structural growth (up to 17 days old), as young males showed shorter tarsus length when they had high contamination levels, although this relationship was not found in young females. During the second part of their development, this relationship was weaker and reversed between the sexes. Whatever their sex, chicks with a high sum of concentrations (among the most prevalent pesticides) had delayed body condition, although this effect was observed only for the first stage of their growth. However, we did not detect any relationship between contamination levels and the mass gain or the physiological stress of nestlings.

Growth rates are mediated through the energy provided by food intake and hormones (McNabb, 2007). Thus, highly contaminated nestlings might need to invest energy in the detoxification process, leading to trade-offs at the expense of growth, and/or might have altered hormone secretions, which in turn alter growth. Although for corticosterone secretion the pattern is clear (i.e., no effect of pesticide contamination levels), for the effects of pesticide mixtures on Montagu's harrier chicks' growth, the picture is more complex. Chicks with high contamination levels did not display a particular pattern of corticosterone production. Effects on corticosterone might arise from the combination of multiple stressors and by assessing pesticide contamination levels alone we do not consider other stressors acting in concert (Baos et al., 2006). A previous study conducted on the same population of Montagu's harriers the same year investigated the role of anthropization on the physiological stress of fledglings and showed no relationship between baseline corticosterone and infrastructure density around the nests (Rabdeau et al., 2023). Thus, neither anthropization nor pesticide contamination levels alone explain the variation in baseline corticosterone levels of chicks, supposing that another factor or concurrent factors are responsible for variations in chicks' plasmatic baseline corticosterone. Besides, the lack of effect on corticosterone levels may be due to the sensitivity of this endpoint to pesticide stressors, and we may have observed different responses using other physiological stress endpoints such as ratio of heterophils to lymphocytes or reactive oxygen species production (Gormally & Romero, 2020; Huber et al., 2021). The oxidative stress for instance would be an interesting endpoint as this could provide information on the potential trade-off in energy expenditure between detoxification processes and hormonal secretions.

Both tarsus length and body condition of Montagu's harrier nestlings seem to be affected during the phase of structural growth (until ~17 days old). In males with high concentrations of pesticides, tarsus length was shorter and although females tended to have larger tarsus with increasing contamination, their tarsus length was still under the tarsus length expected from the general growth curve. This result may be linked to the effects of some pesticides on calcium deposition in bones (Ortiz-Santaliestra et al., 2020). Looking at the three nestlings (one female and two males of less than 17 days old) having the highest contamination loads and delayed tarsus growth, three common pesticides (dicamba, chlorpyrifos-methyl and cypermethrin) are found at concentrations ranging from ~90 to ~655 pg.mg^{-1}. Chlorpyrifos-methyl is an organophosphate insecticide acting as an acetylcholinesterase inhibitor (Table 1). Organophosphates have notably been shown to cause skeleton malformations and stunted growth in birds' embryos (Fry, 1995). Besides, cypermethrin (a pyrethroid insecticide acting as a sodium channel modulator; Table 1) has been shown to cause structural abnormalities and growth retardation in chicks exposed *in ovo* to a mixture with chlorpyrifos (an organophosphate similar to chlorpyrifos-methyl) (Khan, 2020). Consequently, the concomitant presence of chlorpyrifos-methyl and cypermethrin in highly contaminated nestlings that display lowered skeletal growth suggests that the experimental evidence of the adverse effects of these molecules on the growth of birds might occur *in natura*. The herbicide dicamba that acts on plants' cell division and growth has been shown to cause DNA damages, oxidative stress and to act as an endocrine disruptor in vertebrates (Zhu et al., 2014; Soloneski et al., 2016; Ruiz de Arcaute et al., 2019) but no study to our knowledge investigated its potential sublethal effects on birds.

Despite the absence of effect on body mass gain, the fact that higher pesticide load resulted in lowered body condition for both females and males during the structural growth period, supposes that these chicks may have more difficulties accessing food items, integrating nutrients and/or storing fat reserves. Reduced access to food items may be induced by altered foraging activities, as some pesticides that target the nervous system (such as acetamiprid, carbaryl, chlorpyrifos-methyl and thiamethoxam) can result in behavioural changes (Berny et al., 1999; Eng et al., 2017). Although Montagu's harrier nestlings are fed by adults, their behaviour within the nest may influence sibling competition for food and consequently, the amount of food ingested. Moreover, reduced food intake can occur because of the loss of appetite due to pesticide contamination, notably pyrethroid insecticides such as cypermethrin (Sharaf et al., 2010). The disrupting effect of some pesticides on hormone secretions and metabolic pathways could reduce nutrient absorption in the gastrointestinal tract and fat deposition in tissues (Kouamo et al., 2021a). Both *in ovo* exposure and oral ingestion of cypermethrin (alone or in mixture) reduced body weight gain in a dose-dependent manner (Sharaf et al., 2010; Khan, 2020; Kouamo et al., 2021b). Thus, its detection in the three nestlings with the highest pesticide load (within the 10-17 days-old stage) and with poorer body condition than expected adds evidence for the adverse sublethal effect of cypermethrin on birds. Fenpropidin was also a common fungicide in these three nestlings; however, there is no study to our knowledge concerning the sublethal effects of this molecule on birds. Because gut microbiota is involved in nutrient uptake (Grond et al., 2018), this may be the underlying link between pesticides contamination and altered body mass. For instance, in house sparrows (*Passer domesticus*), gut microbiome altered with antibiotics resulted in depressed nestling growth (Kohl et al., 2018). Therefore, an interesting prospect would be to investigate the potential effects of pesticides on the nestlings' gut microbiota.

The contrasting effects between early growth stage and later stage, may be due to the energy expenditure for detoxification processes. Indeed, as observed in another study, the contamination levels of chicks are reduced with age, probably due to a better metabolisation and excretion capacities later in their development (Fuentes et al., 2024a). This suggests that for nestlings going through a crucial developmental period, the cost of detoxification is probably high and at the expense of structural growth and energy storage. It appears thus that nestlings may be particularly vulnerable to the sublethal effects of pesticide mixtures within the first weeks post-hatching and that later in the rearing period they may catch up with the delayed growth. This result is in line with previous findings on the skeletal growth of grey partridge nestlings from parents fed with conventional grains (i.e., containing pesticide residues) (Gaffard et al., 2022), although the time to catch-up size was greater in Montagu's harrier. Nonetheless, this catch-up is very likely to be to the detriment of other life-history traits (immunity or colouration of secondary sexual traits for instance) as for grey partridge nestlings (lower body condition; Gaffard et al., 2022). In addition, the lack of a global trend of the effects of pesticide cocktails may be explained by the fact that trade-offs in energy expenditure are not at the expense of nestling's mass gain or physiological stress but of other life-history traits. This has been notably found in grey partridge chicks which had lower haematocrit levels when body condition was higher at the end of the growth period (Gaffard et al., 2022). Similarly, red-legged partridge chicks from parents fed with contaminated seeds had a reduced immune response compared to control chicks, while their growth was unaffected (Lopez-Antia et al., 2015b). Moreover, the effects of pollutants on the development of nestlings may also be investigated through different biomarker endpoints such as thyroid and growth hormones, and vitamin A (see Rolland, 2000 for a review). These appeared in various studies as better indicators of thyroid disruption than body weight or skeletal growth, which are relatively insensitive and require sustained periods of thyroid deficiency to be affected (McNabb, 2007). However, in European shag nestlings, hepatic levels of POPs were neither associated with circulating thyroid hormones nor hepatic vitamin A despite their effect on wing bone length (Jenssen et al., 2010). High pollutant concentrations during the embryonic development (i.e., present in the egg and yolk) might be more influential on the morphological development post-hatching than those persisting in chicks' organs and/or originating from dietary intakes (Jenssen et al., 2010), which may explain the mismatch between the measured contamination levels and the observed traits. The egg characteristics (size, mass, shell-thickness) and content (yolk and albumen composition) are also crucial for the embryonic and post-hatching growth (Blom & Lilja, 2004; Krist, 2011). Moreover, the nutritional quality of the diet (i.e., the content in vitamin A and other elements) is important to ensure regular levels of hormones and functions despite the effects of pollutants during the embryonic phase (Jenssen et al., 2010).

Consequently, the contamination levels of chicks during their embryonic phase and food quality during their rearing period might blur the lines, counterbalancing the potential link between contamination levels and growth. Still, the titration of pesticides in eggs would suppose to sacrifice the embryo as no technique to collect samples without altering the egg is available to date (Pacyna-Kuchta, 2022). Thus, only abandoned and unhatched eggs could be used to investigate the potential teratogenic effects of pesticide mixtures on Montagu's harriers.

5. Conclusions

We found the first on-field evidence that cocktails of pesticides induce slight alterations in wild nestlings' growth although there is no general pattern. Younger nestlings and males seemed to be more sensitive to the sublethal effects of pesticides. Thus, it appears that the age and sex of chicks are key characteristics to consider when assessing the adverse effects of pesticide mixtures on wild farmland birds. Moreover, even though a catch-up in growth can occur over the rearing period, more investigations are needed to determine if this is at the expense of other life-history traits or not. From the present study at least, there is no evidence that it could be at the expense of stress hormones as we did not find any effect of pesticide mixtures on corticosterone secretion. Thus, an interesting prospect would be to include a wider range of multiple life-history traits (including behavioural traits) to study the sublethal effects of multiple pesticides on Montagu's harrier nestlings. Finally, although we cannot assert the generalization of our findings, our study provides pieces of knowledge into the adverse effects of pesticide cocktails on wild farmland birds *in natura*.

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Supplementary materials

Table S1. Analytical methods (GC = ATD-GC-MS/MS; LC = LC-MS/MS) and limits of detection (LODs) and of quantification (LOQs) in pg.mg^{-1} for the 18 compounds detected in the blood of Montagu's harrier (*Circus pygargus*) chicks. The percentage of detection corresponds to the number of samples with detection of the substance divided by the 70 analysed samples multiplied by a hundred. Compounds and % detection in bold are characterised as the most prevalent molecules.

Compounds	*Type*/ Family	Method	LODs	LOQs	% detection
Acetamiprid	*Insecticide* / Neonicotinoid	LC	0.010	0.034	15.7
Aclonifen	*Herbicide* / Diphenylether	GC	0.044	0.147	8.6
Bupirimate	*Fungicide* / Pyrimidine	GC	0.022	0.075	15.7
Carbaryl	*Insecticide* / Carbamate	GC	0.028	0.102	25.7
Chlorpyrifos-methyl	***Insecticide* / Organophosphate**	**GC**	**0.028**	**0.093**	**61.4**
Cypermethrin	*Insecticide* / Pyrethroid	GC	0.001	0.004	32.9
Dicamba	*Herbicide* / Benzoates	GC	0.124	0.436	24.3
Dichlobenil	*Herbicide* / Nitrile	GC	0.015	0.051	5.7
Ethofumesate	*Herbicide* / Benzofuran	GC	0.007	0.024	27.1
Fenpropidin	***Fungicide* / Piperidine**	**GC**	**0.007**	**0.025**	**48.6**
Fenpropimorph	*Fungicide* / Morpholine	GC	0.003	0.009	20.0
Metamitron	***Herbicide* / Triazinone**	**GC**	**0.058**	**0.192**	**40.0**
Metolachlor-S	***Herbicide* / Chloroacetamide**	**GC**	**0.010**	**0.032**	**35.7**
Penconazole	*Fungicide* / Triazole	GC	0.011	0.039	17.1
Picloram	***Herbicide* / 6-Chloropicolinates**	**GC**	**0.027**	**0.090**	**45.7**
Propazine	*Herbicide* / Triazine	GC	0.070	0.380	14.3
Prosulfocarb	*Herbicide* / Thiocarbamate	GC	0.098	0.358	17.1
Thiamethoxam	*Insecticide* / Neonicotinoid	LC	0.012	0.041	1.4

Table S2. Effects of (i) the total number of pesticides (N_{tot}), sex and age on the residuals of chicks' tarsus and weight growth, and condition through the Scale Masse Index, SMI; and (ii) the N_{tot}, sex, age, brood size and hatching order on the residuals of chicks' BASECORT, REPCORT and MAXCORT. The significance of each variable in the LMMs was tested using a likelihood-based χ^2 test.

Variables	χ^2	df	P
TARSUS			
Intercept	0.832	1	0.362
N_{tot}	0.027	1	0.871
Sex	0.386	1	0.534
Age	1.601	1	0.206
N_{tot} x Sex	0.150	1	0.699
N_{tot} x Age	0.255	1	0.614
Sex x Age	1.242	1	0.265
N_{tot} x Sex x Age	1.135	1	0.287
WEIGHT			
Intercept	0.321	1	0.571
N_{tot}	0.001	1	0.971
Sex	1.021	1	0.312
Age	1.837	1	0.175
N_{tot} x Sex	0.467	1	0.494
N_{tot} x Age	0.556	1	0.456
Sex x Age	3.028	1	0.082
N_{tot} x Sex x Age	2.792	1	0.095
SMI			
Intercept	0.516	1	0.473
N_{tot}	0.131	1	0.717
Sex	0.296	1	0.587
Age	0.431	1	0.512
N_{tot} x Sex	0.114	1	0.735
N_{tot} x Age	0.070	1	0.792
Sex x Age	0.306	1	0.580
N_{tot} x Sex x Age	0.120	1	0.729
BASECORT			
Intercept	0.075	1	0.785
N_{tot}	0.038	1	0.845
Sex	0.444	1	0.505
Age	0.090	1	0.764
Brood size	0.491	1	0.483
Hatching order	1.284	1	0.257

N_{tot} x Sex	0.647	1	0.421
N_{tot} x Age	0.194	1	0.660
Sex x Age	0.480	1	0.488
N_{tot} x Sex x Age	0.844	1	0.358
REPCORT			
Intercept	1.145	1	0.285
N_{tot}	1.832	1	0.176
Sex	0.137	1	0.711
Age	1.695	1	0.193
Brood size	0.753	1	0.386
Hatching order	0.037	1	0.848
N_{tot} x Sex	0.180	1	0.672
N_{tot} x Age	1.587	1	0.208
Sex x Age	0.075	1	0.784
N_{tot} x Sex x Age	0.090	1	0.765
MAXCORT			
Intercept	0.858	1	0.354
N_{tot}	1.074	1	0.300
Sex	0.003	1	0.956
Age	0.986	1	0.321
Brood size	0.193	1	0.660
Hatching order	0.060	1	0.807
N_{tot} x Sex	0.000	1	0.989
N_{tot} x Age	0.894	1	0.344
Sex x Age	0.011	1	0.916
N_{tot} x Sex x Age	0.014	1	0.907

Table S3. Effects of (i) the number of pesticides among the most prevalent (N_{preval}), sex and age on the residuals of chicks' tarsus and weight growth, and condition through the Scale Masse Index, SMI; and (ii) the N_{preval}, sex, age, brood size and hatching order on the residuals of chicks' BASECORT, REPCORT and MAXCORT. The significance of each variable in the LMMs was tested using a likelihood-based χ^2 test.

Variables	χ^2	df	P
TARSUS			
Intercept	0.296	1	0.587
N_{preval}	2.031	1	0.154
Sex	0.437	1	0.509
Age	0.061	1	0.805
N_{preval} x Sex	0.969	1	0.325
N_{preval} x Age	0.434	1	0.510
Sex x Age	0.115	1	0.735
N_{preval} x Sex x Age	0.025	1	0.875
WEIGHT			
Intercept	0.253	1	0.615
N_{preval}	1.458	1	0.227
Sex	0.064	1	0.801
Age	0.420	1	0.517
N_{preval} x Sex	0.412	1	0.521
N_{preval} x Age	0.021	1	0.884
Sex x Age	0.700	1	0.403
N_{preva}l x Sex x Age	0.322	1	0.571
SMI			
Intercept	0.016	1	0.901
N_{preval}	0.306	1	0.580
Sex	1.068	1	0.301
Age	0.003	1	0.956
N_{preval} x Sex	0.825	1	0.364
N_{preval} x Age	0.220	1	0.639
Sex x Age	0.760	1	0.383
N_{preval} x Sex x Age	0.509	1	0.475
BASECORT			
Intercept	0.015	1	0.903
N_{preval}	0.147	1	0.701
Sex	0.143	1	0.705
Age	0.430	1	0.512
Brood size	0.988	1	0.320
Hatching order	0.841	1	0.359

N_{preval} x Sex	0.164	1	0.686
N_{preval} x Age	0.495	1	0.482
Sex x Age	0.105	1	0.745
N_{preval} x Sex x Age	0.137	1	0.711
REPCORT			
Intercept	0.024	1	0.876
N_{preval}	0.132	1	0.717
Sex	0.045	1	0.833
Age	0.104	1	0.747
Brood size	0.450	1	0.502
Hatching order	0.093	1	0.760
N_{preval} x Sex	0.028	1	0.867
N_{preval} x Age	0.083	1	0.773
Sex x Age	0.006	1	0.937
N_{preval} x Sex x Age	0.003	1	0.958
MAXCORT			
Intercept	0.001	1	0.981
N_{preval}	0.002	1	0.961
Sex	0.035	1	0.851
Age	0.018	1	0.892
Brood size	0.006	1	0.939
Hatching order	0.015	1	0.903
N_{preval} x Sex	0.034	1	0.853
N_{preval} x Age	0.032	1	0.858
Sex x Age	0.048	1	0.827
N_{preval} x Sex x Age	0.043	1	0.835

Table S4. Effects of the total sum of pesticide concentrations (∑tot, square root transformed), sex and age on the residuals of chicks' tarsus and weight growth, and condition through the Scale Masse Index, SMI; and (ii) the ∑tot (square root transformed), sex, age, brood size and hatching order on the residuals of chicks' BASECORT, REPCORT and MAXCORT. The significance of each variable in the LMMs was tested using a likelihood-based χ^2 test. Significant effects are depicted in bold.

Variables	χ^2	df	P
TARSUS			
Intercept	**4.362**	**1**	**0.037**
∑tot	1.420	1	0.233
Sex	1.851	1	0.174
Age	**6.287**	**1**	**0.012**
∑tot x Sex	1.338	1	0.247
∑tot x Age	2.448	1	0.118
Sex x Age	**4.002**	**1**	**0.045**
∑tot x Sex x Age	**4.332**	**1**	**0.037**
WEIGHT			
Intercept	1.902	1	0.168
∑tot	0.471	1	0.492
Sex	1.857	1	0.173
Age	2.069	1	0.150
∑tot x Sex	1.246	1	0.264
∑tot x Age	0.225	1	0.635
Sex x Age	2.542	1	0.111
∑tot x Sex x Age	2.029	1	0.154
SMI			
Intercept	0.352	1	0.553
∑tot	0.026	1	0.872
Sex	0.177	1	0.674
Age	1.373	1	0.241
∑tot x Sex	0.065	1	0.799
∑tot x Age	0.688	1	0.407
Sex x Age	0.000	1	0.984
∑tot x Sex x Age	0.063	1	0.802
BASECORT			
Intercept	0.078	1	0.780
∑tot	0.001	1	0.972
Sex	0.001	1	0.972
Age	0.026	1	0.871
Brood size	0.212	1	0.645

Hatching order	1.443	1	0.230
∑tot x Sex	0.000	1	0.997
∑tot x Age	0.061	1	0.804
Sex x Age	0.046	1	0.830
∑tot x Sex x Age	0.061	1	0.805
REPCORT			
Intercept	0.185	1	0.667
∑tot	0.522	1	0.470
Sex	1.813	1	0.178
Age	0.544	1	0.461
Brood size	0.488	1	0.485
Hatching order	0.013	1	0.910
∑tot x Sex	1.767	1	0.184
∑tot x Age	0.501	1	0.479
Sex x Age	1.597	1	0.206
∑tot x Sex x Age	1.334	1	0.248
MAXCORT			
Intercept	0.148	1	0.701
∑tot	0.266	1	0.606
Sex	2.281	1	0.131
Age	0.383	1	0.536
Brood size	0.134	1	0.714
Hatching order	0.144	1	0.704
∑tot x Sex	2.079	1	0.149
∑tot x Age	0.413	1	0.520
Sex x Age	1.688	1	0.194
∑tot x Sex x Age	1.225	1	0.268

Table S5. Effects of the sum of pesticide concentrations among the most prevalent (∑preval, square root transformed), sex and age on the residuals of chicks' tarsus and weight growth, and condition through the Scale Masse Index, SMI; and (ii) the ∑preval (square root transformed), sex, age, brood size and hatching order on the residuals of chicks' BASECORT, REPCORT and MAXCORT. The significance of each variable in the LMMs was tested using a likelihood-based χ^2 test. Significant effects are depicted in bold.

Variables	χ^2	df	P
TARSUS			
Intercept	3.820	1	0.051
∑preval	0.901	1	0.343
Sex	0.568	1	0.451
Age	**6.765**	**1**	**0.009**
∑preval x Sex	0.112	1	0.738
∑preval x Age	2.514	1	0.113
Sex x Age	2.778	1	0.096
∑preval x Sex x Age	2.006	1	0.157
WEIGHT			
Intercept	0.031	1	0.860
∑preval	1.141	1	0.285
Sex	0.023	1	0.879
Age	0.093	1	0.761
∑preval x Sex	0.319	1	0.572
∑preval x Age	0.568	1	0.451
Sex x Age	0.031	1	0.861
∑preval x Sex x Age	0.053	1	0.818
SMI			
Intercept	**4.762**	**1**	**0.029**
∑preval	3.114	1	0.078
Sex	0.128	1	0.721
Age	**6.949**	**1**	**0.008**
∑preval x Sex	0.209	1	0.648
∑preval x Age	**5.083**	**1**	**0.024**
Sex x Age	0.944	1	0.331
∑preval x Sex x Age	1.226	1	0.268
BASECORT			
Intercept	1.350	1	0.245
∑preval	2.955	1	0.086
Sex	0.680	1	0.410
Age	1.414	1	0.234
Brood size	0.185	1	0.667

Hatching order	1.174	1	0.279
∑preval x Sex	0.310	1	0.577
∑preval x Age	1.795	1	0.180
Sex x Age	0.040	1	0.841
∑preval x Sex x Age	0.030	1	0.863
REPCORT			
Intercept	0.421	1	0.516
∑preval	0.945	1	0.331
Sex	0.434	1	0.510
Age	0.949	1	0.330
Brood size	0.504	1	0.478
Hatching order	0.055	1	0.814
∑preval x Sex	0.211	1	0.646
∑preval x Age	0.939	1	0.333
Sex x Age	0.231	1	0.631
∑preval x Sex x Age	0.053	1	0.817
MAXCORT			
Intercept	0.006	1	0.937
∑preval	0.009	1	0.923
Sex	0.044	1	0.835
Age	0.106	1	0.745
Brood size	0.094	1	0.759
Hatching order	0.036	1	0.849
∑preval x Sex	0.043	1	0.836
∑preval x Age	0.069	1	0.793
Sex x Age	0.122	1	0.727
∑preval x Sex x Age	0.113	1	0.737

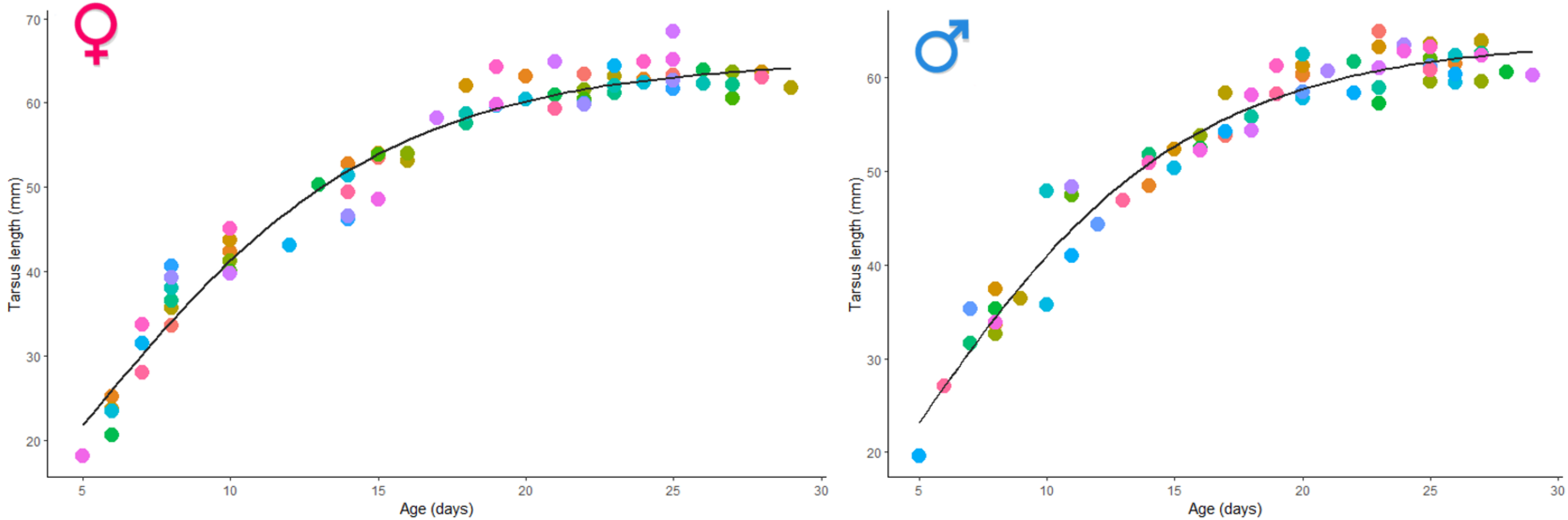

Figure S1. Growth curves of tarsus length (in millimetres) of Montagu's harrier chicks – females (17) on the left side and males (18) on the right. Each colour of dots corresponds to an individual so that multiple morphological measures (2 to 5) are available per chick at different ages. Curves were fitted using a non-linear regression (Gompertz model).

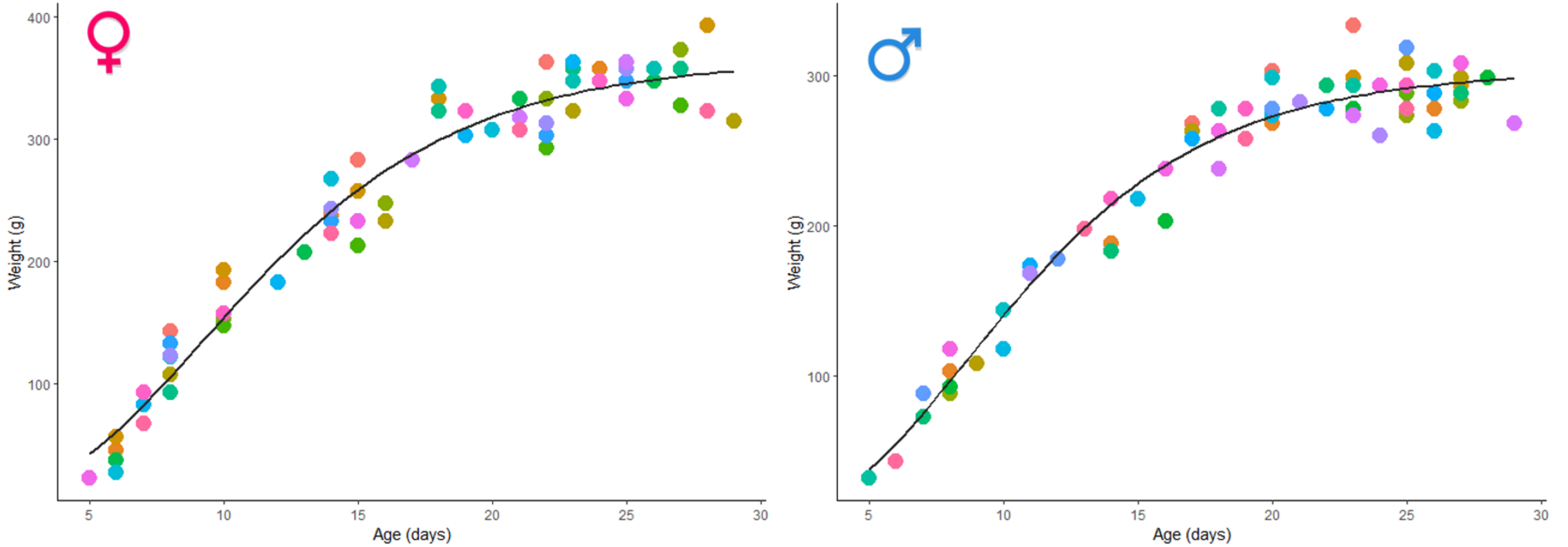

Figure S2. Growth curves of weight (in grams) of Montagu's harrier chicks – females (17) on the left side and males (18) on the right. Each colour of dots corresponds to an individual so that multiple morphological measures (2 to 5) are available per chick at different ages. Curves were fitted using a non-linear regression (Gompertz model).

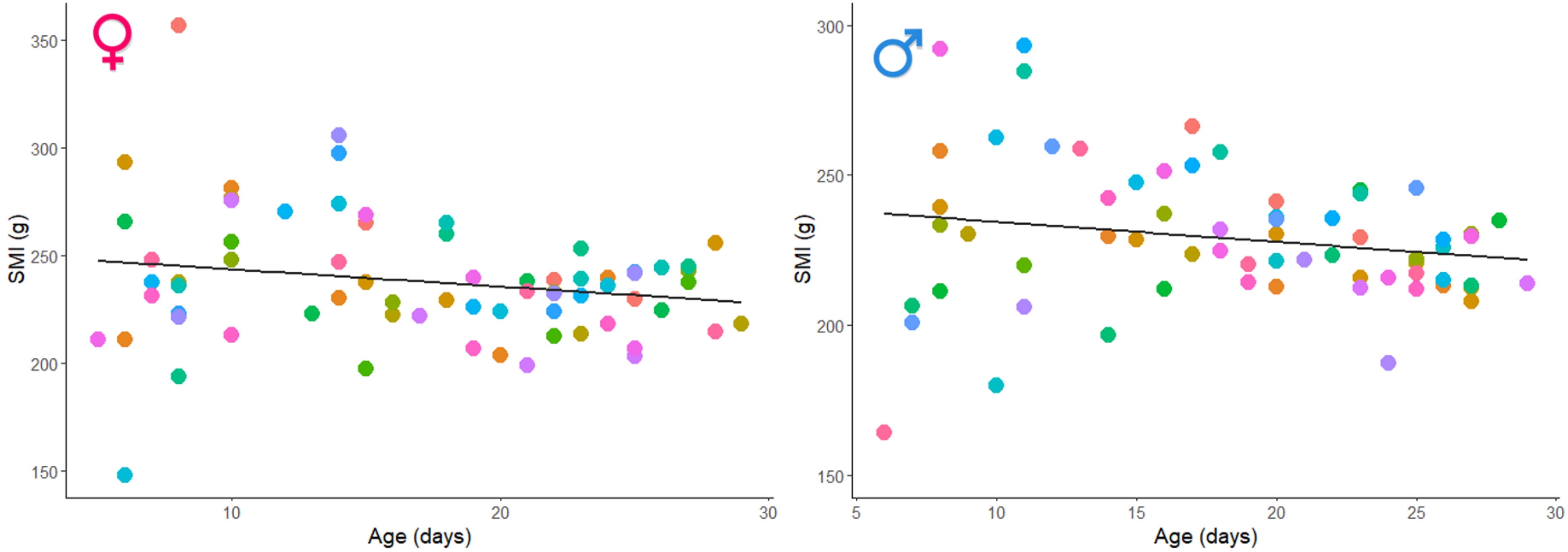

Figure S3. Change in Scale Mass Index (SMI, in grams) of Montagu's harrier chicks with age – females (17) on the left side and males (18) on the right. Each colour of dots corresponds to an individual so that multiple measures (2 to 5) are available per chick at different ages. Models were fitted using a linear regression.

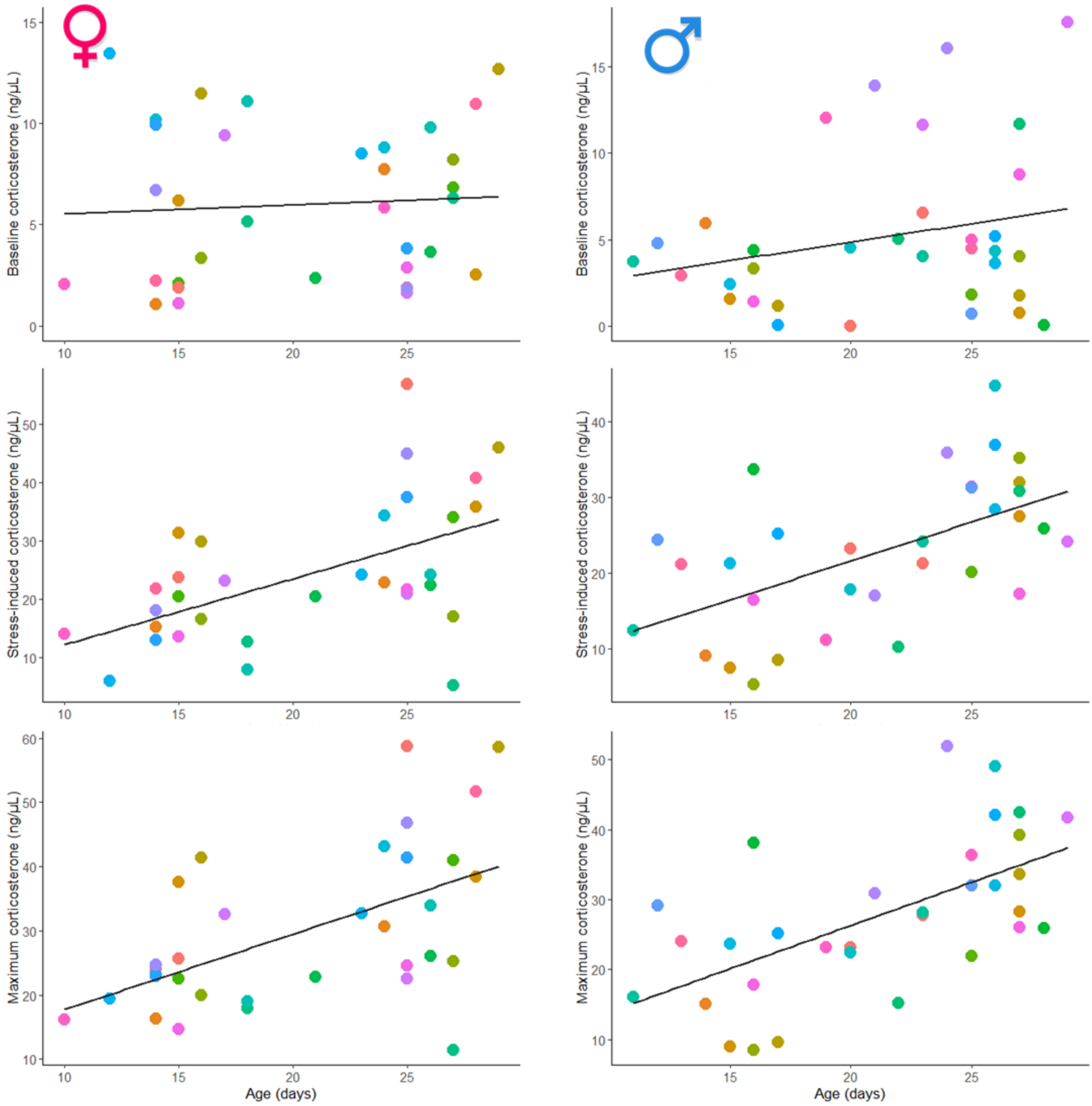

Figure S4. Change in baseline (top), stress-induced (middle) and maximum (bottom) corticosterone (in $ng.\mu L^{-1}$) of Montagu's harrier chicks with age – females (17) on the left side and males (18) on the right. Each colour of dots corresponds to an individual so two measures are available per chick at different ages. Some samples were not available so that sample sizes are 34 females and 34 males for baseline corticosterone, 33 females and 32 males for stress-induced and maximum corticosterone. Models were fitted using a linear regression.

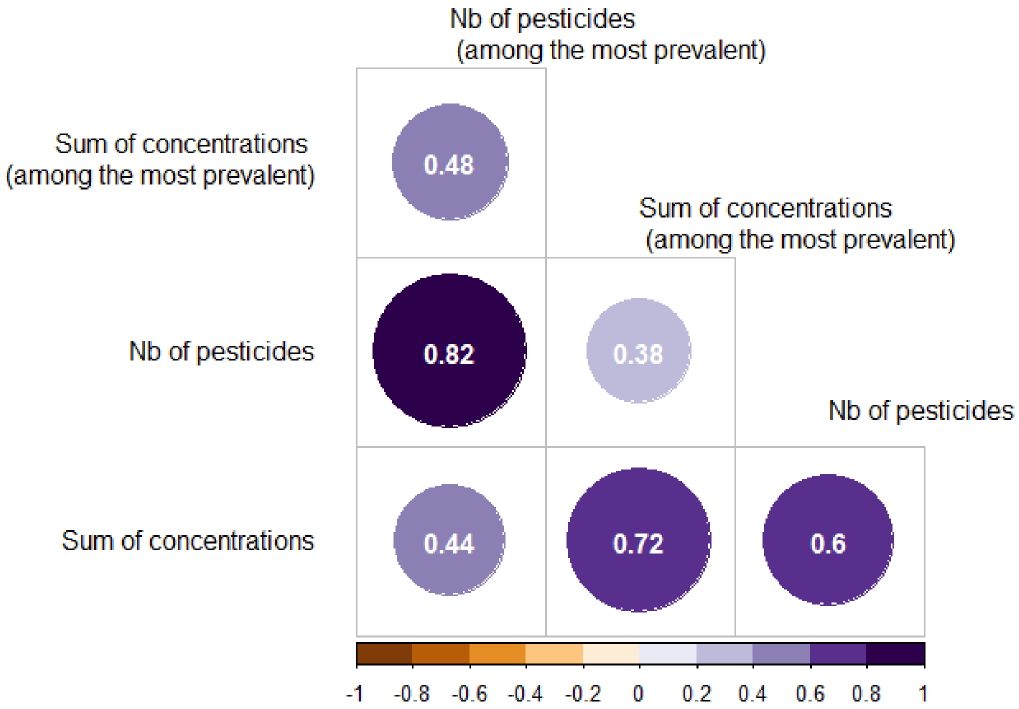

Figure S5. Pearson's correlation coefficients for the four metrics of pesticide contamination levels (35 chicks). Dot colour represents the direction of the correlation (purple = positive, orange-brown = negative) and dot size is proportional to effect size (larger dots = larger effects).

GENERAL DISCUSSION

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Wild species face multiple stressors contributing to populations' decline and, ultimately, local or global extinctions. However, some stressors are more complicated to integrate into estimations and predictions on biodiversity loss as their effects on species are poorly understood. This is the case of pesticide cocktails, for which important knowledge gaps exist on their prevalence and effects in wild species and notably farmland birds (see section "*III.4. Knowledge gaps from pesticide's contamination and sublethal effects on farmland birds in natura*"). My research work participated in filling these knowledge gaps and I provide in this general discussion (i) a summary of the main results obtained from the different chapters presented; (ii) a discussion on how the partial picture given by a conventional vs. organic farming point of view is complemented by multiresidue analyses of pesticides; (iii) a discussion on the first answers to the numerous questions brought by this new body of knowledge (iv) a prospect of non-persistent pesticides in the light of what is known about legacy pesticides; and (v) general conclusions about the implications of my research work on biodiversity and human's health in a *One Health* framework.

I. Overview of the main results

The research I undertook during these last three years provides, through an innovative approach, the first assessment of the relative impacts of organic farming (organic farming) practices on birds' health through the study of multiple life-history traits. Investigating the effects of a gradient of organic farming around Montagu's harrier nests on twelve different proxies of chicks' condition resulted in contrasted conclusions (**CHAPTER I**, *ARTICLE 1*). The relative effects of organic practices may be both positive (enhanced colouration of secondary sexual traits) and negative (increased physiological stress) for nestlings and these influenced organisms at multiple spatial scales. These observations would not have been possible without a large sample size obtained from long-term population monitoring and a fine-scale knowledge of the territory thanks to the LTSER ZAPVS. As organic farming practices mainly differ from conventional practices in their absence of synthetic inputs (the other practices are not mandatory), these findings suggest that synthetic pesticides and fertilisers might be the main underlying factors of the observed effects. However, in the absence of technical means to support it, this can only be supposed as one among other underlying mechanisms.

At the same time as I began my thesis, a novel multiresidue analysis technique was developed by Rodrigues et al. (2023), which allowed me to investigate this question at a new level of resolution by screening up to 116 different compounds, including herbicides, fungicides, insecticides, a safener and a synergist in the blood of wild Montagu's harrier nestlings. This technique allows for the first time to my knowledge, unravelling the extent of the contamination of wild birds and the complexity of the underestimated cocktail effects. I showed that all nestlings (in 2018 and 2021) were contaminated with at least one pesticide during their growing period (**CHAPTER II**). Moreover, the concentrations reported were generally far higher than concentrations reported in species of lower trophic levels or raptors from other studies. These results highlighted the potential biomagnification occurring along the trophic chain, leading to the heavy contamination of Montagu's harrier chicks and questioned the effects on their health.

Although inter-annual differences in pesticide and concentrations were expected, the first striking result was the heterogeneity of contamination patterns among nestlings from the same nest. Our investigations (*ARTICLE 3*) revealed that the sampling of a randomly chosen chick would lead to erroneous conclusions about pesticide loads because nestlings from the same nests were as different in their contamination levels as nestlings from different nests. Moreover, assessing the potential factors influencing pesticide mixtures in chicks allowed me to observe that neither the sex nor the hatching rank of nestlings could explain their pesticide contamination loads (*ARTICLE 3*). These results suggest that other factors (behaviour, individuals' antioxidant contents or metabolisation potential) may influence within nests differences in contamination levels. Besides, I took advantage of the previous monitoring design (2018) consisting of two blood samples at the middle and end of the rearing period to evidence that pesticide contamination levels decreased with increasing age of chicks but that this was unrelated to the sampling date, or their body condition (*ARTICLE 4*). This suggests that other mechanisms were probably involved, such as the development of gut microbiome, and/or better metabolism and excretion capacities in older nestlings. Moreover, organic farming was found to be a key factor influencing the number of pesticides detected in chicks' blood (*ARTICLE 3*), suggesting that organic farming around nests may mitigate the risks associated with pesticide cocktails.

One of the major goals of my work was to investigate the sublethal effects of the detected pesticide mixtures on wild Montagu's harrier nestlings. As previously mentioned, growth parameters, physiological stress and pesticide contamination levels of nestlings might be of interest. Based on the two blood samples from 2018 chicks, I assessed the effects of contamination levels, nestling age and sex on chicks' tarsus growth, mass gain, body condition, and corticosterone secretion. No major pattern was evidenced, but rather an age- and sex-dependent effect of contamination levels on tarsus growth and body condition only (**CHAPTER III**, *ARTICLE 5*). The first stage of the developmental period of chicks seemed to be a sensitive period, especially for males, with a greater risk of adverse effects on growth, although it may be caught up by the end of their development. Therefore, these results provide new evidence of the potential *in natura* sublethal effects of pesticide mixtures on wild farmland birds and emphasise the complexity of considering such effects in field studies.

Overall, the different studies carried out during my doctoral work filled major knowledge gaps concerning pesticide contamination and sublethal effects on birds *in natura*. This new body of knowledge is discussed in the following sections.

II. From a dichotomy to the integration of pesticide mixtures

Organic farming has been shown to provide a range of benefits for biodiversity in agroecosystems, although some studies did not find any effect or even negative effects depending on the taxa and landscape contexts for instance (see Stein-Bachinger et al., 2021 for a review). Concerning farmland birds, most studies realised pairwise comparisons of conventional vs. organic farms and reported increased richness, diversity and abundance in organic farms (Chamberlain et al., 1999; Freemark & Kirk, 2001). Those including the effects of the proportion of organic farming rather than only a paired comparison generally used a defined surface or radius (Goded et al., 2018). Besides, agricultural systems in farmland landscapes have been mostly investigated for their attractiveness to species and rarely for their underlying effects on organisms that may ultimately determine population growth or decline (Latimer et al., 2020). For instance, passerine birds captured in 'organic hedges' (i.e., organic crops accounting for 73–98% of the 250 m radius buffer area), displayed a higher number of flee attempts, handling vigour, pecking and distress calls than birds from 'conventional hedges' (Moreau et al., 2022b). This might be linked to the neurotoxic effects of some pesticides applied in conventional farms and would have ultimate consequences on the population's dynamics as a less intensive behavioural activity may reduce the chances to escape predation and thus increase mortality (Moreau et al., 2022b). Grey partridges fed with conventional grains (containing pesticide residues) showed reduced flight initiation distance and flight duration and were more likely to escape by running than flying which further illustrates the greater risk of predation for birds exposed to pesticide mixtures (Gaffard et al., 2022b). In organic farms, the stress response of birds was generally lower on more locally diverse farms and in landscapes with higher amounts of semi-natural cover (Latimer et al., 2020). Although these studies assessing the effects of organic and conventional farming systems considered different biomarkers within the stress response and different endpoints of behavioural response, no study to my knowledge assessed multiple life-history traits of birds encompassing morphology, physiology and behaviour. Therefore, I provided (*ARTICLE 1*) new insights on the potential influence of organic farming practices on farmland birds' life-history traits, and on how this production system may affect wild populations. In that sense, my work brought new knowledge and insights to the scientific field of agroecology. Still, this remains an incomplete picture as the underlying mechanisms explaining the observed effects of organic farming have not been assessed. Indeed, I did not disentangle the relative effects of synthetic pesticide inputs and of other practices that differ between conventional and organic farming (notably the proportion of natural and semi-natural habitats).

To go further concerning the effects of organic farming practices on nestlings' health, taking into account the time since the conversion to organic would be interesting. The abundance of butterflies has been shown to increase (Jonason et al., 2011), non-marketable ecosystem services to increase (Fan et al., 2018) and soil pesticide contamination to decrease (Riedo et al., 2021) with increasing number of years under organic farming. Besides, as the effects of organic farming practices vary depending on the landscape context (Smith et al., 2020), considering the crop types surrounding nests, the proportion of hedgerows and the proximity to urban areas would have been useful. Notably, the inclusion of the complexity of the landscape could help to better understand the higher physiological stress of nestlings surrounded by higher proportions of organic farming. Including all this information would provide further clarifications concerning the underlying mechanisms of the observed effects of organic farming surrounding Montagu's harrier nests. Apart from a better characterization of the landscape, fine-scale information on the use of synthetic inputs in crops obtained from questionnaires performed with farmers would provide valuable information.

The development of new analytical methods (Rodrigues et al., 2023) to determine the actual contamination with pesticides of farmland birds has been a huge improvement. Although samples of the 380 nestlings included in the first chapter were not available to determine their contamination levels, this analytical method permits to add a piece to the puzzle. Indeed, the determination of pesticide mixtures in wild farmland birds not only adds on-field evidence of the ubiquity of pesticides within agroecosystems but also highlights the role of synthetic pesticides in the observed effects of farming systems on farmland birds. Because my work revealed that organic farming reduces the cocktail of pesticides found in Montagu's harrier nestlings (*ARTICLE 3*), this suggests that the effects observed when the proportion of organic farming around nests was lowered may be due, at least in part, to the higher contamination levels of nestlings. Therefore, can we suppose that increasing the proportion of organic farming in agroecosystems benefits farmland birds and at what proportion/scale can we observe these beneficial effects? Could it tackle their actual population decline? From the results of my work, it is not clear if the benefits of a higher proportion of organic farming would be purely positive on farmland birds' health. The picture is neither black nor white, and future considerations of the effectiveness of organic farming or any farm management for biodiversity conservation should properly investigate its relative effects on multiple traits at different spatial scales.

The improvement provided by the pesticide multiresidue analysis also raises various questions. The detection of pesticides from multiple classes applied to a wide range of crops and non-cropped areas, and some banned for years, questions the exposure pathways of naïve nestlings, hatched on the ground of local cereal crops. It also questions the generality of the findings to other farmland birds and other contexts. This especially raises the question of whether the currently used "non-persistent" pesticides are following in the same footsteps as POPs according to the picture depicted here.

III. First answers to the complexity of pesticide mixtures in farmland birds

Wild birds have been repeatedly shown to be contaminated with multiple pollutants, and within the few studies considering pesticides, most focused on POPs or non-persistent pesticides from a class of interest such as neonicotinoid insecticides or triazole fungicides (Humann-Guilleminot et al., 2021; Fernández-Vizcaíno et al., 2023). For instance, raptor contamination has been mainly assessed for organochlorine insecticides and anticoagulant rodenticides (Gómez-Ramírez et al., 2014). Because nothing was previously known about the factors influencing non-persistent pesticide mixtures or their effects on farmland birds, first simplistic approaches were needed to better understand their decline. Therefore, during my three-year research work, I used metrics and approaches commonly used in ecotoxicology studies on other contaminants such as POPs or heavy metals, on the assumption that non-persistent pesticide mixtures would exhibit similar patterns and effects, but can we draw general conclusions on the results obtained? What concerns can we have about the methodology followed?

1. Can we draw general conclusions…

a. …about the contamination routes?

The determination of pesticide mixtures in Montagu's harrier nestlings evidenced the contamination of farmland birds with pesticides from multiple classes sometimes at rather high concentrations. A remaining question is the relative contribution of each exposure pathway, as these cannot be asserted. It is difficult to define the exposure pathways and doses associated with the observed contamination levels found in wild birds, especially given the lack of data about pressure-to-exposure and metabolisation of pesticides in field conditions. Nevertheless, an interesting way of providing reliable information on the pathways leading from spreading to contamination would be to collect data from questionnaires conducted with farmers. Indeed this constitutes valuable information on the type, time and dose of application. Combined with data on the landscape features (hedgerows, crop types, urban areas), this could be an integrative approach to assess the relative contribution of each exposure pathway to the contamination of wild birds with pesticides. For instance, knowing that a substance has been applied in a sunflower field a few days before nestlings were sampled in the neighbouring cereal crop and that this substance was found in these nestlings but not in nestlings sampled the same day in another neighbouring field separated by a hedgerow would suppose that spray drift resulting in exposure through inhalation pathway has been avoided by the presence of the hedgerow. However, this pressure-to-exposure scenario would be complicated to assert in the absence of replicates, thus compiling information would need to be coupled with the titration of pesticides in other matrices. Soil, vegetation and nest material samples could be collected at the nest to assess the contact-mediated pathway. The use of passive air samplers could be useful to investigate the pesticides present in the atmosphere at nests and thus assess the inhalation exposure pathway (Al-Alam et al., 2021). A promising way to investigate the part of the dietary contamination route would be the titration of pesticides from food pellets collected at nests. Besides, sampling adults' feathers could help to directly identify pesticides that may be present in their wintering areas. Coupled with the titration of pesticides in unhatched eggs collected, this could provide direct evidence of the maternal transfer of non-persistent pesticides *in natura*.

b. …about other farmland birds?

The observations and conclusions drawn for Montagu's harrier may not apply to all farmland birds. Indeed, this apex predator is mostly exposed to pesticides through bioaccumulation and biomagnification processes (Badry et al., 2020), contrary to other taxa of granivorous or insectivorous farmland birds that are potentially more directly exposed to pesticides through their feeding habits. Besides the influence of the chemical properties and toxicokinetics of a pesticide on its potential for bioaccumulation/biomagnification, its effects on the life-history traits of organisms and the detoxification abilities of the contaminated organism also contribute to the relative bioaccumulation/biomagnification of the substance (Tison et al., 2024). Therefore, if we speculate that Montagu's harrier nestlings have greater detoxification potential and/or are less sensitive to the effects of pesticides, the observed patterns may not apply to other farmland birds with lower detoxification capacities and/or more sensitive (or conversely if nestlings have a lowered metabolisation/ are more sensitive). Even among raptors some differences in the metabolism of some compounds exist (Kuo et al., 2022), therefore, further studies including other raptor species within the study area would be valuable. Wild grey partridges captured in the study area in 2020-2022 were heavily contaminated, and 14 of the 50 pesticides detected were common to the 28 substances detected in Montagu's harrier nestlings sampled in 2021 (Bariod et al., 2024). Thus, there seems to be a gap between the contamination of the granivorous and the predator species which could also be due to the sampling period (winter vs. spring/summer) and the age of the birds. Sampling partridge chicks feeding upon insects and adults Montagu's harriers may provide more comparable information. The titration of pesticide mixtures in passerine birds captured within the same area would further help to understand the transfer of pesticides along the trophic chain. The trophic structure and complexity of communities could influence the transfer of pesticides across species and food webs (Tison et al., 2024). Therefore, what is found for Montagu's harriers in an intensively farmed cereal plain may not be the same in another food web, especially if its main prey is different (for instance in central Spain where it feeds upon insects and lagomorphs mainly). Spatial variations of pesticide contamination in birds are rarely investigated within the same study (but see Badry et al., 2022a; 2022b) and are generally indirectly addressed by comparing the results of multiple studies. However, studies conducted in different regions usually differ in the analytical methods used and their comparison is sometimes unwise. Future investigations should thus use equivalent analytical methods to consider farmland birds' contamination with pesticide mixtures from multiple regions under varied contexts. In the current state of the art, because we lack information concerning other wild farmland birds, we cannot assert the generalization of our findings. The first step to fill the mentioned knowledge gaps would be to complete the picture that emerges for the ZAPVS, by the titration of pesticide mixtures in passerine birds and other raptor species. This would provide a global vision of pesticide transfer within a terrestrial food web. Then, this picture would need to be confirmed or denied by assessing pesticide mixture (using the same technique to be comparable) from other terrestrial food webs in contrasted contexts (more extensive farming for instance).

2. Methodological concerns

To improve biomonitoring and effect assessments of pesticide mixtures, we can rationally wonder how well-suited is the methodology used in the present work. Are Montagu's harriers good biosentinels? Is blood a good biological matrix? Are the analyses of data sufficiently accurate?

a. Is Montagu's harrier a good biosentinel species?

Bioindicators are organisms used to assess qualitatively biotic responses to environmental stress (for instance, their presence reflects the presence of a pollutant) while biomonitors are organisms used to determine quantitatively a response (their morphological or physiological condition may indicate the severity of the pollution) (Holt & Miller, 2010). Biosentinels or sentinel species are organisms used to detect the risk to humans by providing early signals of a danger, because they respond first to any change in the environment, for instance to the presence of a pollutant (National Research Council (U.S.), 1991). Although quite different in their definitions, bioindicators, biomonitors and biosentinels share several characteristics. First, they should provide a measurable response which means they should be sensitive to the pollutant (decreasing numbers in relation to the pollutant) or accumulate pollutants (to monitor body burdens and non-lethal effects over the long term). Montagu's harrier being a bird of prey, should provide measurable responses to pollutants due to their accumulation in tissues. In the context of currently used pesticides, because they are less likely to bioaccumulate/biomagnify than legacy pesticides, apex bird predators may not be the first choice biosentinels (Badry et al., 2020). Moreover, any experimentation must be conceived following guidelines for the ethical use of animals in research, and for protected raptor species such as the Montagu's harrier, with even greater caution. However, birds of prey may allow to identify substances that accumulate/magnify and present adverse effects in upper-chain organisms despite their supposed lower risk. Therefore, if the aim is to prioritise substances that require particular attention among the tens (hundreds) to which farmland birds are exposed, then, birds of prey are key biosentinels. Second, biosentinels/bioindicators should be abundant and common so that their distribution overlaps the studied area, they can be easily enumerated and captured, and their harvesting for monitoring has no major adverse impact (although with the present non-lethal techniques available, this last point is not necessarily relevant; National Research Council (U.S.), 1991; Holt & Miller, 2010). The widespread distribution of Montagu's harrier within the region of interest (Europe for instance) makes it a well-suited biosentinel (Badry et al., 2020). Although the species is a long-distance migrant and does not meet the criteria of residency which is supposed to limit or prevent bird exposure to the contaminants of interest (Badry et al., 2020), the exposure of nestlings occurs at the localization of the nest and they are thus presumed to reflect local contamination. Besides, chicks are easily accessible, and although adults are quite more complicated to capture it does not pose safety issues, unlike some raptors whose habitat is hardly accessible. Therefore, Montagu's harrier may be considered a good biosentinel species because it can be easily enumerated and captured. Besides, good bioindicators are well-studied organisms with economic/commercial importance, for instance, a species that raises public interest or awareness (Holt & Miller, 2010), which is the case of Montagu's harrier due to its conservation status and the singularity of its reproduction that attracts public attention. Moreover, it has other advantageous characteristics to biomonitor pesticides: it is an active hunter and inhabits agricultural habitats. The species mainly prey upon rodents and invertebrates, both sedentary prey, so prey/predator exposure to pesticides is co-located, which would eventually not be the case if feeding mostly upon migratory passerines (Badry et al., 2020). Although the present work suggests that maternal transfer may lead to nestling contamination with pesticides used out of the region of interest, further investigations are needed to assert that contamination with banned molecules is not due to their persistence or fraudulent use. Overall, the use of Montagu's harrier chicks as biosentinels of pesticides in agroecosystems provides key information on the risk pesticides pose to farmland birds, especially migrant birds, and further promotes the need for global legislation on pesticide use.

A species that eventually gathers all the advantages of the Montagu's harrier without the downsides mentioned above is the Hen harrier *Circus cyaneus* because this species has a partial migration, is resident in some European countries, and is even more widely distributed in Europe than *C. pygargus*. Besides, *C. cyaneus* has a wider niche compared to other harrier species and may therefore be exposed to a larger range of pesticides including through both terrestrial and freshwater food webs. Nonetheless, because it includes a larger part of avian prey in its diet, this could also induce its contamination with pesticides from uncertain origins. Therefore, all species used as biosentinels present advantages and drawbacks, and their selection should be done according to their ecology and the current state of knowledge.

b. *Is blood a good matrix?*

The tissues sampled in birds to detect contaminants have distinctive related functions: circulation and disposition (blood/plasma), storage and partitioning (fat), metabolism and detoxification (liver), and potential for biomagnification (muscle/flesh) (Kuo et al., 2022). Moreover, these matrices may reflect recent (blood/plasma) to long-term uptake (liver) of pollutants (Espín et al., 2016; Kuo et al., 2022). The advantages and drawbacks of each biological matrix have been repeatedly reviewed (Espín et al., 2016; Kuo et al., 2022; Pacyna-Kuchta, 2023), and blood has been pointed out as the most effective non-destructive method for large-scale biomonitoring, reflecting short-term exposure (few hours to days) and allowing to measure additional endpoints related to sublethal effects (Espín et al., 2016). Despite some disadvantages of the sampling technique (invasive, need of a licence), blood is a valuable matrix to account for pesticide mixture contamination in a short timeframe. However, it is unclear how long this timeframe extends (how many hours/days) for complex mixtures in wild birds and how the remobilization of pesticides from internal tissues influences blood contaminants, notably during migration and egg-laying periods. It has notably been suggested that sampling the blood of older chicks (almost fledging) allows the observation of mainly dietary exposure to pesticides rather than maternal transfer (Badry et al., 2022a). This supposes that within a month, pesticides transferred from the female would be cleared from the nestling's organism or at least undetected in blood. From my work (*ARTICLE 4*), this assumption is partly supported. Montagu's harrier nestlings had reduced pesticide contamination levels with their increasing age, suggesting a higher load in younger chicks due to females' deposition of substances in eggs. However, each pesticide molecule and mixture for a given individual may have a different temporal pattern, probably due to the rate of metabolisation and excretion of complex mixtures within the organism, something that has been ignored in most studies that focused on single molecules. The picture is thus complex, and further studies are needed to determine whether some of the pesticides detected in older chicks can originate from maternal transfer to the egg (especially molecules banned for decades). Therefore, the concomitant collection and analysis of other matrices such as unhatched eggs, feathers or internal tissues from carcasses of wild farmland birds are required to better understand the transport and distribution of pesticides via the blood and thus to determine the relevant timeframe for blood sampling according to the study objectives. The development of technical means for field studies is promising for these future considerations of pesticide fate *in natura*. For instance, passive blood sampling of incubating females using blood-sucking insects in artificial eggs (Becker et al., 2006) and multiresidue analyses using faeces and feathers are potential ways to obtain greater information on spatial and temporal variations of pesticide contamination of wild birds with minimal impact.

c. How to account for the complexity in analyses?

The development of analytical methods, including a wide range of pollutants, is a key element to assess the adverse effects of mixtures on wild organisms. However, these methods bring along the complex task of including a large set of pollutants in statistical analyses. The two metrics used in my work to synthesise pesticide mixtures (i.e., the number of pesticides detected and the sum of pesticide concentrations) are commonly used in ecotoxicology studies and assume an additive effect of pollutants. This means that these metrics assess cocktail effects on birds' health based on the strong assumption that a higher number of compounds or total concentrations increase the number of potential interactions among mixture components and thus the risk of cocktail effects (Fritsch et al., 2022; Zaller et al., 2022). For instance, the toxic unit is a common metric based on this concentration addition principle that estimates a mixture's toxicity by summing each substance's toxic unit (EFSA, 2012). Each individual toxic unit is defined as "the ratio between the concentration of a mixture component and its toxicological acute (e.g. LC50) or chronic (e.g. long-term NOEC) endpoint" (SCHER/SCENIHR/SCCS, 2012). However, these metrics ignore the potential synergistic or antagonist interactions between the molecules of the mixture (Hernández et al., 2017) and do not allow us to observe the relative influence of each pesticide molecule on bird's health. Besides, the use of toxicological acute or chronic endpoints poses problems of availability and relevancy for wild birds, notably raptors, because these are obtained from laboratory model species (quails, partridges or mallards) and chronic endpoints are not available for all substances even in model species (see **Table A1** in Appendices). Soon, novel tools are expected to assess and predict hazards related to realistic mixtures at environmentally relevant doses of pesticides (for further details refer to Hernández et al., 2017). However, in the absence of means that allow us to consider such interacting effects, additivity (using toxicological endpoints or not) remains the main conservative approach used in ecotoxicological studies. A suitable approach would be, for instance, to compare directly pesticides with life-history traits. The effects of pesticide mixtures or any other environmental factors are not directed towards a single life-history trait but rather induce contrasted effects depending on the trait. Therefore, future research should include a wide range of life-history traits to be able to witness potential trade-offs and have a global view of the effects on farmland bird's health. Therefore, an interesting analytical procedure would consider both a complex matrix of pesticides detected/quantified and a complex matrix of life-history traits measured on wild birds. A further improvement in the analytical procedure would be the way to account for the censored data, i.e., the values below detection and quantification limits. Indeed, for the calculation of contamination levels, the values below LOD were substituted by 0 and the values below LOQ by the molecule's LOQ divided by 2. Substitution methods are a good way to handle left-censored observations (Tekindal et al., 2017) although more sophisticated methods exist, these may have some disadvantages and may require specialised software so they are not widely used (Hites, 2019). Nonetheless, datasets with a censoring rate over 65% may induce substantial deviations in the results obtained for mean, median and standard deviation so pesticides detected in less than 35% of samples could potentially be taken off the matrix analysed to provide reliable estimates (Tekindal et al., 2017).

IV. Are currently used pesticides following behind POPs?

1. Do we find similar patterns?

Contrary to studies reporting the effects of the intrinsic factors such as the hatching rank, sex, age and body condition of nestlings on their levels of contamination to POPs (Becker & Sperveslage, 1989; Bustnes et al., 2013), I did find only an effect of the age of nestlings while none of the other factors considered affected contamination levels. Therefore, my work clearly shows that non-persistent pesticides do not follow the same processes as those observed in POPs, so we do not observe the same biological patterns. This calls for further investigations to find which factors could determine the high intra-brood variability of contamination levels. Concerning inter-brood variability, the role of the nest location and surrounding habitat is important for non-persistent pesticide exposure as shown for POPs (Eulaers et al., 2013). Nonetheless, other land uses than organic farming and elements of the habitat deserve further investigations to complete the picture.

Few field studies addressed temporal variations of pesticide contamination in wild birds, and these focused on intra- and/or inter-annual changes in organochlorine insecticides (exclusively POPs, Ormerod & Tyler, 1994; Mañosa et al., 2003; Gómez-Ramírez et al., 2019). Since attention to non-persistent pesticides is more recent, biomonitoring schemes and tools are less developed than for POPs. Nonetheless, long-term monitoring studies integrating a larger selection of pesticides will probably develop in the upcoming years (see for instance Peris et al., 2023). Wild Montagu's harrier nestlings sampled for three different years (2018 in *ARTICLE 4*; 2021 in *ARTICLE 2*; 2022 in *ARTICLES 7 and 8 in Appendices*) provide the first observations on the temporal variation of non-persistent pesticide contamination in wild farmland birds. In fact, among the 18 substances detected in 2018, 28 in 2021 and 38 in 2022, only two pesticides were detected in all three years (metamitron and cypermethrin). Because the proportion of dominant crop types in the study area is relatively constant across the years (Bretagnolle et al., 2018b), the type of pesticides applied should stay relatively constant. Consequently, either the spatial distribution of nestlings or the pesticide inputs applied to the main crops have changed between 2018 and 2021. Montagu's harrier nests are not necessarily located within the same fields across the years but colonies are generally found in the same sectors of the study area every year (see Fig. 1 in ARTICLE 1). Consequently, a spatial change is unlikely to explain inter-annual differences in pesticides detected. Wild partridges captured in two consecutive winters within the same study area also displayed yearly variations of non-persistent pesticide contamination, although some molecules were detected during both winters (Bariod et al., 2024). Therefore, yearly changes in the pesticides applied to crops may explain the observed pattern. The restriction of use and ban of some molecules for instance could result in lower concentration and/or non-detection in the following years. However, substances such as neonicotinoids have been banned since 2018 in the study area and are still found in farmland birds in 2021 and 2022 (*ARTICLES 7 and 8 in Appendices*). Moreover, chlorpyrifos-methyl was detected in 61% of the blood samples from 2018, has been banned in

Europe along with chlorpyrifos in 2020 and was undetected in the 55 samples collected in 2021. This could suggest that the prohibition was effective, however, the detection of chlorpyrifos in chicks sampled in 2022 questions its illegal use and/or persistence. It seems thus that these pesticides are probably not following a straightforward trend as can be found for most legacy organochlorine pesticides, i.e., a reduction in contamination levels found in wild birds across the years since their prohibition (see however Mañosa et al., 2003 for an example of increased concentrations of POPs). This highlights the need for long-term biomonitoring to get a clear view of temporal variations, and to assert if these non-persistent pesticides follow the same trends POPs did or not. Other environmental factors influencing the exposure of chicks (weather or food abundance for instance) that were not assessed in my work could also be responsible for inter-annual variations and deserve further investigation. Although being a specialist raptor, the part of alternative prey in the Montagu's harrier diet varies between years and along the breeding season according to their main prey availability. These prey vary in their antioxidant content (involved in detoxification processes), thus, temporal variation in food items could modulate pesticide contamination as observed for temporal trends of POPs related to changes in the diet (Mañosa et al., 2003). The weather could also make the pesticide contamination vary greatly in the environment (Zaller et al., 2022) and have consequences on chicks' health (inclement weather could have adverse effects on food abundance and predation risk; Berger-Geiger et al., 2019), resulting in variations of contamination levels. Thus, future studies are needed to disentangle the role of these factors on wild farmland birds' contamination.

2. A large persistence?

The presence of banned pesticides in the blood of nestlings sampled in different years (**Chapter II**) is an additional observation to previous studies detecting legacy pesticides in different environmental compartments of the study area (Pelosi et al., 2021; Fritsch et al., 2022). Nonetheless, the contamination of wild nestlings is reported for the first time for some prohibited substances (tebutam, for instance, banned in France since 2002). A possible explanation is the maternal contamination route. Montagu's harriers travel long distances during migration and overwinter in western African countries for individuals from the studied population. The legislative framework is different from Europe, banned molecules in France may be completely approved in these countries. Consequently, females may be exposed to substances that are banned in France during their stay and migration stop-overs, then detoxify these molecules during egg-laying, and these would in turn be detected in nestlings. However, among the few studies reporting the presence of banned pesticides in wild birds (Kitowski et al., 2021; Musseau et al., 2023; Bariod et al., 2024; *Article 6 in Appendices*), species as the grey partridge are non-migratory, supposing local contamination (Bariod et al., 2024). Therefore, contamination may result from the fraudulent use of these substances. Indeed, in the study area, cases of illegal use of some compounds have been reported (V. Bretagnolle, pers. com.), thus this explanation cannot be disregarded. Once applied in fields, these banned compounds may contaminate eggs and chicks through the same pathways as currently used pesticides. Apart from fraudulent use, the persistence of pesticides in soil may be an explanation. Some substances can persist for months to years in the environment, thus nestlings can eventually be exposed to banned compounds. Some practices and substances may notably increase soil erosion, releasing pesticides stored in soils (Sabatier et al., 2014). Therefore, even in untreated areas, pesticides have been detected in soil and air/fog water, testifying to the persistence and long-range transport of these substances, even after 20 years of absence of application (Riedo et al., 2021; Zaller et al., 2022; Khoury et al., 2024). Neonicotinoids are a good example of "non-persistent" pesticides that appear to be more persistent than primarily thought. Indeed, neonicotinoids are supposed to have a low persistence as they have short half-lives in both water and soil compartments, spanning from less than a day to a few hundred days (up to 1386 days for clothianidin DT50 in soil; Zhang et al., 2023). However, 3 years after their ban these molecules were still remaining in agroecosystems and detected in farmland birds of multiple trophic levels (*ARTICLE 6 in Appendices*). Remediation strategies are increasingly developed to remove these residues from agricultural soils, notably biodegradation using neonicotinoid-degrading microbes that may produce nontoxic or less toxic metabolites (Zhang et al., 2023). However, given that some parent compounds were supposed to be nontoxic or less toxic to non-target organisms and that this was not fulfilled, we have reasons to question whether these remediation technologies will really be effective and produce safer chemicals. Anyways, the quantification of neonicotinoids and other "non-persistent" pesticides, sometimes at rather high concentrations in Montagu's harrier nestlings is additional evidence for the insidious risk that these substances may pose to birds. The inclusion of more years of biomonitoring and the investigation of yearly environmental factors that may influence the exposure of chicks such as weather or food abundance for instance would have been useful to better understand inter-annual variations in pesticide contamination. Continuing monitoring in upcoming years could help to assess the efficacy of statutory decisions as some molecules, apart from neonicotinoids, have been prohibited during the studied period (fenpropimorph and chlorpyrifos(-methyl) for instance). Temporal trends in pesticide contamination are also useful to assess potential long-term effects on populations (Oli et al., 2023) and deserve thus further investigations.

3. Towards a better or worse case scenario?

Returning to the three characteristics of POPs stated in the introduction, *"(i) a half-life [...] greater than 6 months in soil and sediments and 2 months in water; (ii) a high potential of propagation; (iii) a high bioaccumulation factor and/or toxicity to non-target species"*, from the present research work and other scientific studies, these are applying to some currently used pesticides. Although this work did not investigate abiotic compartments of the environment, the picture provided by the contamination patterns of bioindicators such as the Montagu's harrier and the increasing evidence that currently used pesticides may pose similar environmental and health problems as POPs, suggests that these may follow the same scenario. The huge diversification and internationalisation of pesticides pose serious questions concerning their fate and effects in the upcoming years. Indeed, despite the ban on some hazardous pesticides, a ban is generally followed by the development of at least another less-known pesticide, so we may wonder if we are not assisting a worse scenario. The replacement of organochlorine insecticides by organophosphates, carbamates, pyrethroids and then neonicotinoids is evidence that regulation of some substances is not the only way to tackle their effects because new chemicals enter the market and may pose even greater risks (Tooker & Pearsons, 2021). Maybe the only solution would be to stop the development of new substances until there are reliable methods to assess their real fate in the environment and their effects on non-target organisms under field conditions and accounting for mixtures, including the adjuvants of a formulation. However, this unrealistic solution would not suit the interests of pesticide manufacturers and could worry farmers about the prospects for their production. Therefore, a less extreme way to protect the ecosystems' health would be to promote alternative solutions to the use of synthetic inputs through practices as the Integrated Pest Management (IPM) or biological control. Organic production systems being the extreme solution with no use of synthetic inputs, this alternative mode of production might mitigate the adverse effects of the emerging scenario. Although organic farming has a great role to play in global food and ecosystem security, no one farming system alone would feed the whole planet (Reganold & Wachter, 2016), thus, a first step to protect the ecosystems' health would be to harmonise existing legislation based on the more restrictive procedure, to enforce global regulation systems. Europe has the strictest regulatory system for pesticides and is a key player in the international market; its approach could thus be a base model for global harmonization. International scientific collaborations to enhance the risk assessment procedures of the EFSA rather than conducting individual country research programs would help to fill the knowledge gaps of pesticide risks and establish a harmonized system, to offer a better scenario for the future.

V. General conclusion

My work contributed to filling some of the knowledge gaps in the field of ecotoxicology, adding evidence of the ubiquity of pesticide cocktails in agroecosystems and providing key information for further biomonitoring schemes using farmland birds. It also provided the first evidence of pesticide mixtures' sublethal effects on wild farmland birds and the role of organic farming in mitigating them. It also opened up a large number of questions on the exposure pathways and factors influencing contamination levels of wild birds, and on the way to handle complex ecotoxicological data. Moreover, it left open the question of the underlying mechanisms linking pesticide use to the decline of farmland bird species. Although some answers may be inferred from laboratory studies, there is still a lot of work to determine the complex processes *in natura*. Overall, my work paved the way for future on-field considerations of the effects of pesticide mixture on wild birds. Determining the influence of pesticide cocktails on wild species' health is crucial in a *One Health* context. Indeed, as agreed by the *One health* high-level expert panel (OHHLEP), the health of humans is intimately linked to that of domestic and wild animals, plants and environment including ecosystems (OHHLEP et al., 2022). Therefore, determining how pesticide mixtures affect farmland birds' health could provide new insights into their potential effects on human health. Global harmonization and enforcement of pesticide legislation would therefore benefit not only all human parties involved: pesticide manufacturers (more efficient access to global markets), consumers (enhanced food security), farmers (safer and profitable) and regulators (globally shared knowledge) (Handford et al., 2015), but also the environment and biodiversity in a *One Health* framework.

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APPENDICES

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TABLE A1

LIST OF THE 116 SUBSTANCES INCLUDED IN THE MULTIRESIDUE ANALYSIS

Table A1. Main properties of the 116 substances screened in the multiresidue analysis. Substances are classified by use and then by chemical family. Approval in Europe was obtained from the EU pesticide database (https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances, accessed on October 2024). NA = not applicable, corresponds to substances that have not been assessed at the European level because they are not considered a pesticide active substance of a PPP (synergist, safener or veterinary substances). Bird acute LD50 corresponds to the lethal dose 50% (the quantity killing 50% of the test animals). Bird chronic 21d NOEL corresponds to the no-observed effect level (the highest dose that has no observable effect on the test animals fed for 21 days). Lab species tested in laboratory: *Colinus virginianus* (C.V.), *Coturnix japonica* (C.J.), *Coturnix coturnix japonica* (C.J.), *Anas platyrhynchos* (A.P.) and *Serinus canaria* (S.C.). Log P corresponds to the log of the partition coefficient and measures the lipophilicity of molecules (the larger the value, the more lipophilic). DT50 corresponds to the detection time 50% (time to detect a 50% decrease in pesticide concentration) obtained from field studies or typical (mean of all studies from general literature) if unavailable. Information was compiled from the Pesticide Properties Database (PPDB) of the University of Hertfordshire (http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm, accessed on October 2024), and from HRAC (2024), IRAC (2024) and FRAC (2024). NA values indicate that the information was missing from the PPDB. The analytical method corresponds to either ATD-GC/MSMS (GC) or LC/MSMS (LC), with the limit of detection (LOD) and limit of quantification (LOQ) of each substance analysed.

Name	**Chemical family**	**Use**	**Mode of action**	**Approval in Europe**	**Bird LD50 (mg.kg^{-1})**	**Lab species**	**Birds - Chronic 21d NOEL (mg. kg^{-1} bw d^{-1})**	**NOEL Lab species**	**Log P**	**DT50 (field or typical in days)**	**Analytical method**	**LOD**	**LOQ**
Metalaxyl-M	Acylalanine	Fungicide	Disrupts fungal nucleic acid synthesis (RNA polymerase 1)	Yes	981	C.V.	84	C.V.	1.71	14.1	LC	0.000	0.001
Diphenylamine	Amide	Fungicide	Inhibition of polyene and isoprene biosynthesis	No	>2250	C.V.	NA	NA	3.82	NA	GC	0.001	0.004
Cyprodinil	Anilinopyrimidine	Fungicide	Amino acid and protein synthesis (inhibition of protein synthesis)	Yes	>500	A.P.	64	C.V.	4	45	GC	0.001	0.004
Pyrimethanil	Anilinopyrimidine	Fungicide	Amino acid and protein synthesis (inhibition of protein synthesis)	Yes	>2000	A.P.	95.96	C.V.	2.84	31.4	GC	0.150	0.500
Quinoxyfen	Aryloxyquinoline	Fungicide	Signal transduction (mechanism unknown)	No	>2250	C.V.	NA	NA	5.1	169.3	GC	0.005	0.016

Carbendazim	Benzimidazole-Carbamate	Fungicide	Cytoskeleton and motor proteins (Inhibition of beta-tubulin assembly in mitosis)	No	>2250	C.V.	26.4	A.P.	1.48	22	LC	0.004	0.014
Isopyrazam	Carboxamide	Fungicide	Succinate dehydrogenase inhibitor (SDHI)	No	>2000	C.V.	32.5	C.V.	4.25	72	LC	0.011	0.038
Chlorothalonil	Chloronitrile	Fungicide	Multi-site activity	No	>2000	C.J.	58.2	C.V.	2.94	17.9	GC	0.045	0.135
Procymidone	Dicarboximide	Fungicide	Osmotic signal transduction	No	>4092	C.V.	NA	NA	3.3	208.3	GC	0.016	0.055
Prochloraz	Imidazole	Fungicide	Sterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	No	662	C.V.	33.1	C.V.	3.5	68.8	GC	0.017	0.057
Pyraclostrobin	Methoxy-carbamate	Fungicide	Quinone outside inhibitor (QoI)	Yes	>2000	C.V.	105	C.V.	3.99	33.3	LC / GC	0.011 / 0.125	0.038 / 0.405
Dimethomorph	Morpholine	Fungicide	Cellulose synthesis inhibitor	No	>2000	C.V.	60.4	C.V.	2.68	44	GC	0.007	0.024
Fenpropimorph	Morpholine	Fungicide	Inhibition of sterol biosynthesis in membranes	No	>2000	C.V.	>30.3	C.V.	4.5	25.5	GC	0.003	0.009
Dimoxystrobine	Oximino-acetamide	Fungicide	Quinone outside inhibitor (QoI)	No	>2000	C.V.	36	A.P.	3.59	22.9	GC / LC	0.004 / 0.019	0.013 / 0.064
Kresoxim-methyl	Oximinoacetate	Fungicide	Quinone outside inhibitor (QoI)	Yes	>2150	A.P.	51.7	C.V.	3.4	1.0	GC	0.019	0.063
Trifloxystrobine	Oximino-acetate	Fungicide	Quinone outside inhibitor (QoI)	Yes	>2000	C.V.	31	C.V.	4.5	1.69	GC	0.039	0.132
Fludioxonil	Phenylpyrrole	Fungicide	Osmotic signal transduction	Yes	>2000	C.V.	11.1	Unknown species NOEC	4.12	16	GC	0.037	0.379
Folpet	Phthalimide	Fungicide	Multi-site activity	Yes	>2510	C.V.	>78.3	C.V. NOEC	3.02	3	GC	0.050	0.167
Fenpropidin	Piperidine	Fungicide	Inhibition of sterol biosynthesis in membranes	Yes	1899	A.P.	14.6	C.V.	2.9	49.2	GC	0.008	0.025
Boscalid	Pyridinecarboxamide	Fungicide	Succinate dehydrogenase inhibitor (SDHI)	Yes	>2000	C.V.	25.4	C.V.	2.96	254	GC	0.000	0.002

Bupirimate	Pyrimidine	Fungicide	Nucleic acid metabolism (adenosine-deaminase)	Yes	>10000	C.J.	≥98	C.J.	3.68	34	GC	0.023	0.075
Fenarimol	Pyrimidine	Fungicide	Disrupts membrane function (demethylation inhibitor (DMI))	No	>2000	C.V.	NA	NA	3.69	74	GC	0.001	0.002
Spiroxamine	Spiroketal-amine	Fungicide	Inhibition of sterol biosynthesis in membranes	Yes	565	C.V.	5.4	C.V.	2.89	52.4	GC	0.028	0.094
Azoxystrobin	Strobilurin	Fungicide	Respiration inhibitor (QoL fungicide)	Yes	>2000	C.V.	1200	C.V.	2.5	180.7	GC	0.001	0.003
Tolylfluanid	Sulphamide	Fungicide	Multi-site activity	No	>2000	C.J.	NA	NA	3.9	6	GC	0.008	0.025
Etridiazole	Thiadiazole	Fungicide	Lipid peroxidation inhibitor	No	560	C.V.	NA	NA	3.37	20	GC	0.188	0.625
Zoxamide	Toluamide	Fungicide	Cytoskeleton and Motor Proteins (tubulin polymerization)	Yes	>2000	C.V.	122.8	A.P.	3.76	6	GC	0.024	0.081
Cyproconazole	Triazole	Fungicide	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	No	94	C.V.	1.4	A.P.	3.09	129	GC	0.019	0.064
Difenoconazole	Triazole	Fungicide	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	Yes	>2150	A.P.	9.71	C.V.	4.36	91.8	GC	0.036	0.120
Epoxiconazole	Triazole	Fungicide	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	No	>2000	C.V.	0.91	C.V.	3.3	97.7	LC	0.003	0.009
Flusilazole	Triazole	Fungicide	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	No	>1590	A.P.	NA	NA	3.87	94	GC	0.014	0.048
Myclobutanil	Triazole	Fungicide	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	No	510	C.V.	24.2	C.V.	2.89	35	GC	0.021	0.071
Penconazole	Triazole	Fungicide	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	Yes	>1590	A.P.	28.6	A.P.	3.72	89.7	GC / LC	0.012 / 0.001	0.039 / 0.003

Propiconazole	Triazole	Fungicide	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	No	>2510	A.P.	25.5	A.P.	3.72	35.2	GC / LC	0.001 / 0.001	0.003 / 0.005
Tebuconazole	Triazole	Fungicide	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	Yes	1988	C.V.	5.8	C.V.	3.7	47.1	GC / LC	0.007 / 0.001	0.023 / 0.005
Tetraconazole	Triazole	Fungicide	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	Yes	132	C.V.	NA	NA	3.56	430	GC	0.027	0.091
Triadimenol	Triazole	Fungicide	Ergosterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	No	>2000	C.V.	7.5	C.V.	3.18	36.5	GC	0.053	0.175
Prothioconazole	Triazolinthione	Fungicide	Sterol-biosynthesis inhibitor (demethylation inhibitor (DMI))	Yes	>2000	C.V.	78	A.P.	2	0.77	LC	0.028	0.098
Picloram	6-Chloropicolinates	Herbicide	Auxin mimics	Yes	> 1944	A.P.	65	C.V.	-1.92	33.7	GC	0.027	0.090
Propyzamide	Amide	Herbicide	Inhibition of microtubule assembly	Yes	6578	C.J.	30.9	C.V.	3.27	50.5	GC	0.002	0.007
Tebutam	Amide	Herbicide	Inhibition of microtubule assembly	No	>5000	A.P.	NA	NA	3	60	GC	0.053	0.175
Diclofop-methyl	Aryloxyphenoxy-propionate	Herbicide	Acetyl-CoA carboxylase (ACCase) inhibitor	Yes	> 2250	C.J.	20	C.V.	4.8	19	GC	0.018	0.061
Dicamba	Benzoate	Herbicide	Auxin mimics	Yes	188	C.V.	77	A.P.	-1.80	3.9	GC	0.124	0.436
Ethofumesate	Benzofuran	Herbicide	Inhibition of very long-chain fatty acid synthesis (VLCFA)	Yes	>2000	A.P.	265	C.V.	2.7	37.8	GC	0.007	0.024
Carbetamide	Carbamate	Herbicide	Inhibition of Microtubule Organization	No	>2000	C.V.	169	C.J.	1.78	8	LC	0.005	0.018
Chlorpropham	Carbamate	Herbicide	Inhibition of microtubule organization	No	>2000	C.C.J	94.7	C.V.	3.76	13.1	GC	0.056	0.158
Acetochlor	Chloroacetamide	Herbicide	Inhibition of very long-chain fatty acid synthesis (VLCFA)	No	928	C.V.	NA	C.V.	4.14	12.1	GC	0.028	0.094

Alachlore	Chloroacetamide	Herbicide	Inhibition of very long-chain fatty acid synthesis (VLCFA)	No	1536	C.V.	NA	NA	3.09	14	GC	0.028	0.093
Dimethachlore	Chloroacetamide	Herbicide	Inhibition of very long-chain fatty acid synthesis (VLCFA)	Yes	524	C.C.J.	102	C.J.	2.17	3.2	GC	0.028	0.094
Dimethenamid-P	Chloroacetamide	Herbicide	Inhibition of very long-chain fatty acid synthesis (VLCFA)	Yes	>1068	C.V.	114	C.V.	1.89	15.8	GC / LC	0.028 / 0.007	0.093 / 0.025
Metazachlor	Chloroacetamide	Herbicide	Inhibition of very long-chain fatty acid synthesis (VLCFA)	Yes	>2000	C.V.	76.5	C.V.	2.49	6.8	GC	0.041	0.136
S-Metolachlor	Chloroacetamide	Herbicide	Inhibition of very long-chain fatty acid synthesis (VLCFA)	No	>=2510	A.P.	NA	NA	3.05	23.17	GC	0.010	0.032
Pendimethalin	Dinitroaniline	Herbicide	Inhibition of microtubule assembly	Yes	1421	A.P.	17.5	A.P.	5.4	100.6	GC / LC	0.027 / 0.025	0.089 / 0.083
Trifluralin	Dinitroaniline	Herbicide	Inhibition of microtubule assembly	No	>2250	C.V.	102.85	C.V.	5.27	170	GC	0.008	0.028
Aclonifen	Diphenylether	Herbicide	Inhibition of solanesyl diphosphate synthase (SDPS)	Yes	>2000	C.V.	>141	C.J.	4.37	80.4	GC	0.044	0.147
Bifenox	Diphenylether	Herbicide	Inhibition of protoporphyrinogen oxidase (PPO)	Yes	>2000	C.V.	290	C.J.	3.64	17.3	GC	0.001	0.004
Clomazone	Isoxazolidinone	Herbicide	Inhibition of deoxy-d-xylulose phosphate synthase (DXPS)	Yes	>2224	C.V.	76.5	C.V.	2.58	27.3	GC	0.012	0.040
Dichlobenil	Nitrile	Herbicide	Inhibition of cellulose synthesis	No	698	C.V.	NA	NA	2.7	5.4	GC	0.015	0.051
Flumioxazin	N-Phenylimide	Herbicide	Inhibition of protoporphyrinogen oxidase (PPO)	Yes	>2250	C.V.	34.3	A.P	2.55	17.6	GC	0.042	0.112

Oxadiazon	N-phenyl-oxadiazolone	Herbicide	Inhibition of protoporphyrinogen oxidase (PPO)	No	>2150	C.V.	NA	NA	5.33	165	GC	0.009	0.029
Flurochloridone	Organochlorine	Herbicide	Inhibition of Phytoene Desaturase (PDS)	Yes	>2150	C.V.	NA	NA	3.36	40.6	GC	0.121	0.403
Isoxaflutole	Oxazole	Herbicide	Inhibition of hydroxyphenyl pyruvate dioxygenase (HPPD)	Yes	>2150	A.P.	25	C.V.	2.34	1.3	GC	0.038	0.128
2.4-D	Phenoxycarboxylate	Herbicide	Auxin mimics	Yes	>500	C.V.	>100	C.J.	-0.82	28.8	GC	0.012	0.038
2.4-MCPA	Phenoxycarboxylate	Herbicide	Auxin mimics	Yes	377	C.V.	93.2	C.V.	-0.81	25	GC	0.158	0.526
Mecoprop-P	Phenoxycarboxylate	Herbicide	Auxin mimics	Yes	500	C.V.	70.9	C.J.	-0.19	21	GC	0.050	0.167
Diflufenican	Phenylether	Herbicide	Inhibition of Phytoene Desaturase (PDS)	Yes	>2150	C.V.	91.84	C.V.	4.2	64.6	GC / LC	0.013 / 0.003	0.043 / 0.010
Chlorotoluron	Phenylurea	Herbicide	Inhibition of photosynthesis at photosystem II	Yes	272	C.C.J.	9.6	C.J.	2.5	12.5	LC	0.008	0.026
Diuron	Phenylurea	Herbicide	Inhibition of photosynthesis at photosystem II	No	1104	C.V.	NA	NA	2.87	229	LC	0.004	0.014
Isoproturon	Phenylurea	Herbicide	Inhibition of photosynthesis at photosystem II	No	1401	C.V.	NA	NA	2.5	23	LC	0.002	0.006
Chloridazon	Pyridazinone	Herbicide	Inhibition of photosynthesis at photosystem II	No	>2000	C.V.	21.8	C.V.	1.19	18.6	GC / LC	0.021 / 0.025	0.071 / 0.085
Fluroxypyr	Pyridyloxy-carboxylate	Herbicide	Auxin mimics	Yes	>2000	A.P.	40.1	A.P.	0.04	3	LC	0.035	0.102
Foramsulfuron	Sulfonylurea	Herbicide	Inhibition of acetolactate synthase (ALS)	Yes	>2000	C.V.	104	C.V.	-0.78	25.4	LC	0.004	0.014
Nicosulfuron	Sulfonylurea	Herbicide	Inhibition of acetolactate synthase (ALS)	Yes	>2000	C.V.	171	C.J.	0.61	19.3	LC	0.007	0.024
Triflusulfuron-methyl	Sulfonylurea	Herbicide	Inhibition of acetolactate synthase (ALS)	No	>2250	C.V.	27.6	C.V.	0.94	4.5	LC	0.001	0.004

Prosulfocarb	Thiocarbamate	Herbicide	Inhibition of very long-chain fatty acid synthesis (VLCFA)	Yes	>2250	C.V.	131	A.P.	4.48	9.8	GC	0.098	0.358
Propazine	Triazine	Herbicide	Inhibition of photosynthesis at photosystem II	No	>10000	A.P.	NA	NA	3.95	131	GC	0.070	0.380
Terbutryn	Triazine	Herbicide	Inhibition of photosynthesis at photosystem II	No	>4640	A.P.	NA	NA	3.66	52	LC	0.012	0.038
Hexazinone	Triazinone	Herbicide	Inhibition of photosynthesis at photosystem II	No	>2258	C.V.	NA	NA	1.17	105	GC	0.120	0.610
Metamitron	Triazinone	Herbicide	Inhibition of photosynthesis at photosystem II	Yes	1302	C.J.	81.5	C.V.	0.85	11.1	GC	0.058	0.192
Sulcotrione	Triketone	Herbicide	Inhibition of hydroxyphenyl pyruvate dioxygenase (HPPD)	Yes	>1350	A.P.	NA	NA	-1.7	3.6	LC	0.002	0.007
Lenacil	Uracil	Herbicide	Inhibition of photosynthesis at photosystem II	Yes	>2000	A.P.	100.4	C.V.	1.69	39.8	GC	0.100	0.333
Diflubenzuron	Benzoylphenylurea	Insecticide	Inhibition of chitin biosynthesis affecting CHS1	No	>5000	C.V.	42.7	C.V.	3.89	3	LC	0.010	0.003
Lufenuron	Benzoylurea	Insecticide	Inhibition of chitin biosynthesis affecting CHS1	No	2000	A.P.	NA	NA	5.12	256	LC	0.010	0.035
Flufenoxuron	Benzoylureas	Insecticide	Inhibition of chitin biosynthesis affecting CHS1	No	>2000	C.V.	NA	NA	5.11	42.9	LC	0.011	0.038
Carbaryl	Carbamate	Insecticide	Acetylcholinesterase (AchE) inhibitor	No	>2000	A.P.	NA	NA	2.36	16	GC	0.028	0.102
Fenoxycarb	Carbamate	Insecticide	Juvenile hormone receptor modulator	No	>3000	A.P.	17.7	A.P.	4.07	5.94	GC	0.001	0.004

Pirimicarb	Carbamate	Insecticide	Acetylcholinesterase (AChE) inhibitor	Yes	20.9	C.V.	12.1	A.P.	1.7	9	GC	0.012	0.039
Propoxur	Carbamate	Insecticide	Acetylcholinesterase (AChE) inhibitor	No	>25.9	C.V.	NA	NA	0.14	28	GC	0.098	0.352
Chlorantraniliprole	Diamide	Insecticide	Ryanodine receptor modulators	Yes	>2250	C.V.	>10.1	C.V.	2.86	204	LC	0.001	0.003
Cyantraniliprole	Diamide	Insecticide	Ryanodine receptor modulator	Yes	>2250	C.V.	93.2	C.V.	2.02	32.4	LC	0.111	0.370
Acetamiprid	Neonicotinoid	Insecticide	Nicotinic acetylcholine receptor (nAChR) competitive modulators	No	98	A.P.	9.5	A.P.	0.8	3	LC	0.010	0.034
Clothianidin	Neonicotinoid	Insecticide	Nicotinic acetylcholine receptor (nAChR) competitive modulators	No	430	C.V.	56.8	C.V.	0.905	121.2	LC	0.010	0.034
Dinotefuran	Neonicotinoid	Insecticide	Nicotinic acetylcholine receptor (nAChR) competitive modulators	NA	>2000	C.J.	NA	NA	-0.549	75	LC	0.011	0.037
Imidacloprid	Neonicotinoid	Insecticide	Nicotinic acetylcholine receptor (nAChR) competitive modulators	No	31	C.J.	9.3	C.V. NOAEL	0.57	174	LC	0.015	0.043
Nitenpyram	Neonicotinoid	Insecticide	Nicotinic acetylcholine receptor (nAChR) competitive modulators	NA	1124	A.P.	NA	NA	-0.66	8	LC	0.009	0.030
Thiacloprid	Neonicotinoid	Insecticide	Nicotinic acetylcholine receptor (nAChR) competitive modulators	No	35	S.C.	3.73	A.P.	1.26	8.1	LC	0.001	0.005
Thiamethoxam	Neonicotinoid	Insecticide	Nicotinic acetylcholine receptor (nAChR) competitive modulators	No	576	A.P.	29.4	A.P.	-0.13	39	LC	0.012	0.041
Chlordecone	Organochlorine	Insecticide	Central nervous system stimulant. GABA-gated chloride channel antagonist.	No	237	C.J.	NA	NA	4.5	300	GC	0.270	0.510

Lindane	Organochlorine	Insecticide	GABA-gated chloride channel antagonist	No	122	C.V.	NA	NA	3.5	148	GC	0.035	0.118
Azinphos-ethyl	Organophosphate	Insecticide	Acetylcholinesterase (AchE) inhibitor	No	>12.5	C.J.	NA	NA	3.18	50	GC	0.032	0.076
Chlorfenvinphos	Organophosphate	Insecticide	Acetylcholinesterase (AChE) inhibitor	No	80	C.V.	NA	NA	3.8	30	LC	0.050	0.167
Chlorpyrifos	Organophosphate	Insecticide	Acetylcholinesterase (AChE) inhibitor	No	39.2	C.V.	2.885	A.P.	4.7	27.6	GC	0.017	0.056
Chlorpyrifos-methyl	Organophosphate	Insecticide	Acetylcholinesterase (AChE) inhibitor	No	923	C.V.	NA	NA	4	1.24	GC	0.028	0.094
Malathion	Organophosphate	Insecticide	Acetylcholinesterase (AChE) inhibitor	No	359	C.V.	NA	NA	2.75	1	GC	0.058	0.194
Parathion	Organophosphate	Insecticide	Acetylcholinesterase (AChE) inhibitor	No	2.1	A.P.	NA	NA	3.83	17	GC	0.630	1.300
Indoxacarb	Oxadiazine	Insecticide	Voltage-dependent sodium channel blocker	No	73.5	C.V.	NA	NA	4.65	5.97	GC	0.007	0.023
Tebufenpyrad	Pyrazole	Insecticide	Mitochondrial complex I electron transport inhibitors	Yes	>2000	C.V.	6.6	A.P.	4.93	4.5	GC	0.017	0.056
Bifenthrin	Pyrethroid	Insecticide	Sodium channel modulator	No	1800	C.V.	6.63	C.V.	6.6	86.8	GC	0.003	0.012
Cypermethrin	Pyrethroid	Insecticide	Sodium channel modulator	Yes	>9520	A.P.	4.29	C.V.	5.55	21.9	GC	0.001	0.004
Deltamethrin	Pyrethroid	Insecticide	Sodium channel modulator	Yes	>2250	C.V.	>55	C.V.	4.6	21	GC	0.001	0.003
lambda-Cyhalothrin	Pyrethroid	Insecticide	Sodium channel modulator	Yes	>3950	A.P.	3.3	A.P.	5.5	26.9	GC	0.024	0.081
Pymetrozine	Pyridine	Insecticide	Chordotonal organ TRPV channel modulator	No	>2000	A.P.	21.8	C.V.	-0.19	22.6	LC	0.022	0.073
Spinosad-A	Spinosyn	Insecticide	Nicotinic acetylcholine receptor (nAChR) allosteric modulators site I	Yes	>1333	A.P.	NA	NA	2.8	17.3	LC	0.000	0.001
Spinosad-D	Spinosyn	Insecticide	Nicotinic acetylcholine receptor (nAChR) allosteric modulators site I	Yes	>1333	A.P.	NA	NA	3.2	14.5	LC	0.001	0.003

Benoxacore	Benzoxazine	Safener	Accelerates the detoxification of metolachlor (has no biocidal activity of its own)	NA	NA	NA	NA	NA	NA	NA	GC	0.021	0.067
Piperonyl butoxyde	Benzodoxiole	Synergist	Blocks pests' natural detoxification system. P450-dependent monooxygenase inhibitor.	NA	>2250	C.V.	NA	NA	4.75	13	GC	0.000	0.001

ARTICLE 6

NEONICOTINOIDS: STILL PRESENT IN FARMLAND BIRDS DESPITE THEIR BAN

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Neonicotinoids: Still present in farmland birds despite their ban

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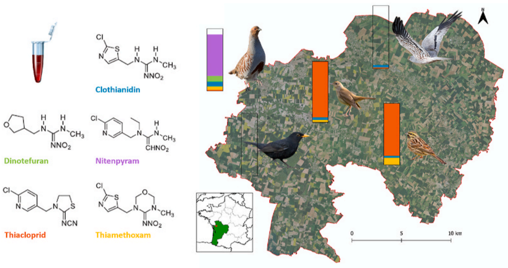
HIGHLIGHTS

- Neonicotinoids (neonics) quantification in blood of wild birds is scarce.
- Neonics are found in passerine birds, grey partridges and Montagu's harriers' blood.
- Clothianidin, thiacloprid and thiamethoxam, banned in France since 2018, are found.
- Dinotefuran and nitenpyram, used in veterinary care, are found in grey partridges.
- Wild fauna exposure questions the persistence of neonics in the environment.

GRAPHICAL ABSTRACT



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Raptors

ABSTRACT

Neonicotinoids (neonics) are the most widely used insecticides worldwide and are considered to be of low risk to non-target organisms such as vertebrates. Further, they are reported to be rapidly excreted and metabolized, reducing their potential toxicity. Nevertheless, growing evidence of adverse effects of neonics on farmland bird species raise questions about the purported harmless nature of these pesticides. We attempted to search for pesticide residues in species of different trophic levels and at different life stages, by using multiple bird monitoring programs on a Long-Term Socio-Ecological Research (LTSER) platform. Three passerine birds—the blackbird (*Turdus merula*), cirl bunting (*Emberiza cirlus*), and common nightingale (*Luscinia megarhynchos*)—that feed on seeds and invertebrates were monitored during their reproductive period, and the grey partridge (*Perdix perdix*) that feeds on seeds was monitored during its wintering period. We also monitored chicks of an apex predator—the Montagu's harrier (*Circus pygargus*)—that preys mostly upon common voles but also upon insects. We found that the birds' blood samples showed presence of residues of five neonics: three banned since 2018 in France—clothianidin, thiacloprid, and thiamethoxam—and two—dinotefuran and nitenpyram—used for veterinary purposes only. While none of these neonics was detected in blackbirds, all were present in grey partridges. Clothianidin was detected in all species, except blackbirds. Concentrations of the three banned neonics were similar or higher than concentrations found in birds monitored elsewhere before the ban. These findings raise questions about the persistence of neonics within the environment and the mode of exposure to wild fauna. Future investigations on the sublethal effects of these neonics on life-history traits of these farmland birds may

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help in providing a better understanding of the effects of exposure of bird populations to these insecticides, and also to the consequent effect on human health.

1. Introduction

During the last century, the need for feeding the growing human population worldwide has led to an intensification of agricultural practices, including an extensive use of pesticides. Despite their use for millennia, several pieces of evidence implicate pesticides in the global decline in biodiversity (Wood and Goulson, 2017; Stanton et al., 2018; Moreau et al., 2022a). Moreover, other studies identify them as the cause of certain diseases in humans (Köhler and Triebskorn, 2013). As all living organisms in a given area share the same environment, protecting biodiversity by reducing pesticides inputs means also reducing humans' exposure to pesticides, and this is necessary to ensure our own health and safety (*One Health* concept). One way is thus to capitalize on wildlife monitoring schemes to better understand the risk of pesticide exposure for humans (Moreau et al., 2022a). In that respect, wild bird species are valuable candidates as they are well-monitored worldwide, being involved in long-term banding programs over decades or more, which have highlighted a global declining trend in several taxa and especially in farmland birds, e.g., in France, Sweden, United Kingdom, US, Europe, and North America (Wretenberg et al., 2006; Comolet-Tirman et al., 2015; Stanton et al., 2018; Rosenberg et al., 2019; Li et al., 2020; Burns et al., 2021; DEFRA, 2021). Pesticide use has been often identified as a major component responsible for this decline (Campbell et al., 1997; Geiger et al., 2010; Mineau and Whiteside, 2013; Chiron et al., 2014; Tassin de Montaigu and Goulson, 2020). Recent studies, for instance, showed a negative relationship between the abundance of northern bobwhites (*Colinus virginianus*) and their exposure to neonicotinoid insecticides (neonics) from 1978 to 2012 (Ertl et al., 2018). Similarly, a wide-panel dataset regarding neonics use and birds' population trends revealed a significant negative impact of this family of pesticides on avian biodiversity from 2003 to 2010 in the Netherlands (Hallmann et al., 2014) and from 2008 to 2014 in USA (Li et al., 2020).

Neonics are insecticides developed in the 1970s, with the first patent dating back to 1977 for nithiazine (rapidly abandoned due to its poor stability), followed by patents for imidacloprid and thiacloprid in 1985, nitenpyram in 1988, acetamiprid and clothianidin in 1989, thiamethoxam in 1992, and dinotefuran in 1994 (Tomizawa and Casida, 2005). Neonics target the neural function and are competitive agonists of nicotinic acetylcholine receptors (nAChR), which increase specificity to insects and not vertebrates (Tomizawa and Casida, 2003). In fact, in contrast to other insecticides such as carbamates and organophosphorous that also target the neural function by inhibiting the acetylcholinesterase (AChE) enzyme—an ubiquitous enzyme in the animal kingdom (review in Grue et al., 1997; Story and Cox, 2001; Walker, 2003; Mitra et al., 2011)—neonics were supposed to have minimal effects and a low toxicological impact on vertebrates and consequently on birds, due to the lower number of nAChRs they have and the lower sensitivity of their nAChRs compared to those of insects (Tomizawa and Casida, 2003, 2005; Ihara et al., 2017; Casida, 2018). Moreover, they were claimed to be rapidly metabolized and excreted, in a few hours (Bishop et al., 2018, 2020; Casida, 2018; Bean et al., 2019; English et al., 2021; Pan et al., 2022), but some reports question their fate along the trophic chain as they have been found in insectivorous birds, granivorous birds, piscivorous birds, and birds of prey (see references in the supplementary materials Table S1). Under experimental conditions, they seem to accumulate in the liver (Lopez-Antia et al., 2015a) and to be detectable in different organs and tissues, although they (at least for imidacloprid and thiamethoxam) seem to be rapidly cleared from birds' organism (Bean et al., 2019; Pan et al., 2022). Several studies showed various effects of neonics exposure on birds at different physiological levels (review in Gibbons et al., 2015 and Moreau et al., 2022a). Although neonics do not act directly on AChE, they may induce neuronal degeneration, which affects AChE activity (Abu Zeid et al., 2019; Rawi et al., 2019), altering more complex functions such as learning and migration behaviour (Eng et al., 2017) but also having sublethal effects on important functions of the organism such as the haematocrit, antioxidant defences, immunity, or fecundity (Lopez-Antia et al., 2013, 2015a; 2015b; Tokumoto et al., 2013; Hoshi et al., 2014; Mohanty et al., 2017; Humann-Guilleminot et al., 2019; Lv et al., 2020).

Since the 1990s, the use of neonics has become widespread, making them the most widely used class of insecticide worldwide, mostly for coating seeds, despite being shown to impact non-target species, including humans (Tomizawa and Casida, 2005; Casida and Durkin, 2013; Gibbons et al., 2015; Simon-Delso et al., 2015; Henry et al., 2015; Wood and Goulson, 2017; Casida, 2018; Thompson et al., 2020; Zhang et al., 2022). In Europe, despite an EU moratorium in 2014, neonics were still detectable in bee-attractive crop nectar until 2018 at least (Wintermantel et al., 2020). Indeed, their degradation in soil (measured by DT_{50} which is the Detection Time 50% representing the time to detect a 50% decrease in pesticide concentration) can take quite a long time, up to more than 6900 days (i.e., 19 years for clothianidin; see Table 2 in Thompson et al., 2020). Therefore, banning harmful neonics does not necessarily eradicate the problem of exposure. In EU, dinotefuran and nitenpyram have never been considered for use in phytopharmaceutical products (PPP; July 2022, EU Pesticides database: https://food.ec.europa.eu/plants/pesticides_en) but are commonly used in veterinary medicine. In France, neonics are banned for outdoor use and in PPP since September 2018 (Décret n° 2018–675, July 2018), except for emergency authorized use of thiamethoxam and imidacloprid on sugar beet crops in production areas (EFSA, 2021). Nonetheless, the use of neonics is still allowed in veterinary medicine.

In the present study, we use different bird monitoring programs on a long-term socio-ecological research (LTSER) platform and a multi-residue analysis (Rodrigues et al., 2023) to evaluate the presence of neonics in an intensive farmland area where acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam were banned for 3 years for agricultural use. Nitenpyram and dinotefuran were also included in the screening to control for potential exposure of wild fauna to veterinary products. As the method was not specifically developed for neonics detection, acetamiprid and imidacloprid, were not detectable among the other 104 pesticide compounds. We selected five different species for their different ecology: (i) three passerine birds during their reproductive period: the blackbird (*Turdus merula*), cirl bunting (*Emberiza cirlus*), and common nightingale (*Luscinia megarhynchos*) that feed on seeds and invertebrates; (ii) the grey partridge (*Perdix perdix*) caught during its wintering period when it feeds only seeds; and (iii) an apex predator species, namely, the Montagu's harrier (*Circus pygargus*) that preys mostly upon common voles but also upon orthopterans. For this fifth species, we focused on chicks that are fed by their parents during the rearing period. Beyond the interest of their contrasted ecologies, farmland bird species including buntings, blackbirds or raptors have been shown to be exposed to neonics, however, a limited number were subject of measures in blood samples (Lennon et al., 2020a). Additionally, grey partridge is recognised to be a focal species for pesticide risk assessment (Millot et al., 2017; Bonneris et al., 2019). Here, we aimed at monitoring potential exposure of multiple bird species in an area where there is presumably no use of neonics for agricultural purposes for 3 years and where nitenpyram used in veterinary medicine has never been assessed to our knowledge. We thus collected blood samples from all individuals to determine the presence and measure the level of exposure to neonics. We selected blood (whole blood, i.e., red blood cells and plasma) for the analyses in order to focus on the effects of short-term exposure only

(Espín et al., 2016), so that potential exposure of the migratory species (i.e., common nightingales, Montagu's harriers, and blackbirds) at wintering areas can be neglected.

2. Materials and methods

2.1. Study area

The study site is located in southwestern France (46°11′N, 0°28′W, Fig. 1), in the Long-Term Socio-Ecological Research Zone Atelier Plaine & Val de Sèvres (LTSER ZAPVS), a 450 km^2 area where the soil occupancy and the agricultural practices are monitored each year since 1994 (Bretagnolle et al., 2018). In this intensive farming area, winter cereal crops accounted for ~41% (wheat: 33.8% and corn: 9.6%) of the area under cultivation; in addition, there were sunflower (10.4%), oilseed rape (8.3%), pea (2%), and meadows (13.5%) (average coverage between 2009 and 2016, Bretagnolle et al., 2018). In this area, organic farming (no pesticide use) is carried out in 18% of the agricultural area. Detailed data on pesticide applications was not available, however, until 2018 imidacloprid was used on cereal crops in the study area, thiacloprid, thiamethoxam and clothianidin for their part were mainly used in maize, oilseed rape and cereal crops. Different monitoring studies performed in this area showed the transfer of neonics in several compartments. For instance, imidacloprid was found in soils, oilseed rape nectar, earthworms, and small mammals in this area (Henry et al., 2015; Wintermantel et al., 2020; Pelosi et al., 2021; Fritsch et al., 2022). Thiacloprid was detected in soils, earthworms and small mammals' hair while thiamethoxam was detected in nectar and soils, and clothianidin in nectar and small mammals' hair (Wintermantel et al., 2020; Pelosi et al., 2021; Fritsch et al., 2022). Acetamiprid has been detected in small mammals' hair (Fritsch et al., 2022) despite its use is mainly for market gardening such as tomatoes, squash, and melon cultures which are not present in a substantial surface of the study area. All five neonics are banned for agricultural use in France since 2018, except imidacloprid and thiamethoxam for emergency authorizations on sugar beet crops. Still, sugar beet crops are only present in this area for their first year (seed production), and the use of neonics is banned for this purpose. Therefore, at the moment when birds were caught (2020–2022, see section 2.2.1), no neonics were being used for agricultural purposes. However, imidacloprid, dinotefuran, and nitenpyram may be in domestic use as veterinary treatment for domestic animals (cats, dogs, ferrets, and bunnies). There is no known screening of nitenpyram in the study area and dinotefuran has only been investigated in one previous study but was not detected in small mammals' hair sampled (Fritsch et al., 2022).

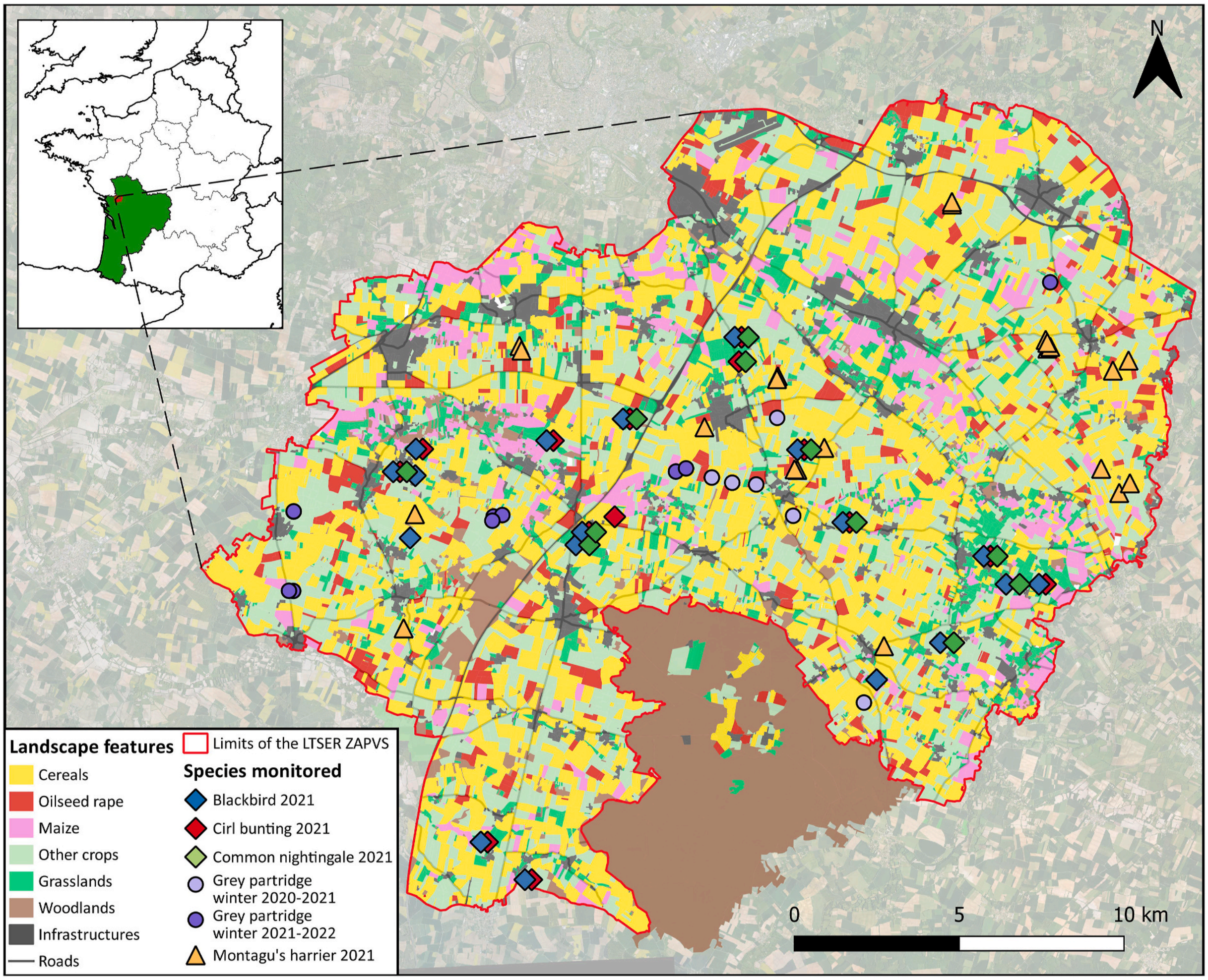

Fig. 1. Spatial distribution of birds' catching sites across the Long-Term Socio-Ecological Research Zone Atelier Plaine & Val de Sèvres (LTSER ZAPVS). Landscape features provided correspond to data available for 2021 from our GIS database. Infrastructures correspond to buildings, sport fields, cemeteries, locks, and bridges present in the study area. The five bird species monitored in the study area are blackbird (*Turdus merula*, $N_{2021} = 64$), cirl bunting (*Emberiza cirlus*, $N_{2021} = 31$), common nightingale (*Luscinia megarhynchos*, $N_{2021} = 34$), grey partridge (*Perdix perdix*, $N_{2020\text{-}2021} = 23$ and $N_{2021\text{-}2022} = 31$), and chicks of Montagu's harrier (*Circus pygargus*, $N_{2021} = 55$). N corresponds to the number of adults sampled, except in the case of Montagu's harrier where N corresponds to the number of chicks sampled.

2.2. Model species and blood collection

2.2.1. Model species

2.2.1.1. Adult passerine birds. The passerine species were sampled during the reproductive period from mid-April to end of June 2021. Birds were caught using net-trapping, following the same methodology as Moreau et al. (2022b). Among the 17 species trapped in this area, we selected three, namely, cirl buntings (N = 31), blackbirds (N = 64), and common nightingales (N = 34) because these species were among the biggest of those captured, which allowed us to draw enough blood for analysis without risking any individual's health. They were also the most caught in the study area allowing us to make a large spatial screening.

2.2.1.2. Adult grey partridges. Grey partridges were caught during two consecutive years from November 2020 to February 2021 (winter 2020–2021) and from December 2021 to March 2022 (winter 2021–2022) in the study area. These individuals, even when caught in the wild, are gamebirds and have been probably raised in captivity before being released for hunting purposes; however, knowing the proportion of captive-born vs. wild-born partridges is almost impossible as banding before release is not mandatory. In winter, grey partridges are mostly herbivorous and granivorous, living in large coveys in winter crops and sleeping in ploughed fields at night. Thermal binoculars were used to spot them at nightfall and birds were then captured in the dark by dazzling them and using a landing net (i.e., a method inspired by Eurasian Woodcock catching technique; Williams, 2015). Blood samples were then collected, and birds were immediately released. A total of 23 and 31 partridges were caught during the 2020–2021 and 2021–2022 winters, respectively.

2.2.1.3. Montagu's harrier chicks. The Montagu's harrier has been monitored in the ZAPVS since 1994 (Bretagnolle et al., 2018). In this intensive agricultural area, they nest mainly on the ground of cereal crops and lay up to eight eggs (Arroyo et al., 1998; Millon et al., 2008). The incubation period lasts 29 days, and the rearing period is between 30 and 35 days (Arroyo et al., 2007). The mean productivity in this site is 2.05 fledglings per breeding attempt (Arroyo et al., 2004), this success depending mainly on the availability of its main prey, the common vole (*Microtus arvalis*) although in case of poor vole availability, harriers may also feed on orthopterans (Salamolard et al., 2000; Butet and Leroux, 2001). Blood samples of chicks that were 26 ± 2 days old were collected from June 2021 to early August 2021. Fifty-five chicks from 22 nests were sampled.

2.2.2. Blood sampling procedure

For all species, blood samples were collected in 2021, and in 2020 and 2022 for grey partridges, on wild individuals included in different monitoring programs (see details in section 2.2.1). For all of them, blood sampling was conducted by puncturing the brachial vein using a sterile needle and using heparinized capillaries to collect 50 μL of blood. Blood samples were placed in Eppendorf tubes and kept refrigerated (0–5 °C), before being returned to the laboratory where they were stored at −20 °C for further analyses.

2.3. Neonics analysis

Neonic extractions were conducted following the method reported by Rodrigues et al. (2023; see also Table S2 in supplementary materials for a description of the neonics). Briefly, blood samples were defrosted and weighed, and a mixture of 2 mL of dichloromethane and ethyl acetate (1:1) was added to each sample, followed by homogenisation by using a vortex for 1 min. Extracts were then sonicated for 10 min. This sonication step was repeated three successive times. After each sonication step, a centrifugation step of 5 min was performed, and supernatants were collected, pooled, and then gently evaporated under a fume hood until a final volume of 500 μL. The extract was collected and stored at −20 °C until the analyses to determine pesticide levels were performed by liquid chromatography coupled to tandem mass spectrometry (LC/MSMS) using multiple reactions monitoring (MRM) for quantification.

LC/MSMS analyses were conducted with a Thermo Scientific TSQ Quantum Access Triple Quadrupole Mass Spectrometer operating in heated positive electrospray ionization mode (HESI+) coupled with a Thermo Accela 1250 pump and a Thermo Combi Pal autosampler. Analyses were performed on a Nucleodur C_{18} Pyramid column (150 mm × 3 mm, 3 μm). Samples were analysed in the gradient mode using a mobile phase composed of water and acetonitrile with both containing 0.1% formic acid.

The multiresidue analysis comprised detection and quantification of 104 pesticide molecules, including five neonics—clothianidin, dinotefuran, nitenpyram, thiacloprid, and thiamethoxam—performed using the MRM detection mode. The source was operated in the positive ionization mode with a spray voltage of 4500 V and the same spray and capillary temperatures of 300 °C each. Nitrogen was used as the sheath and auxiliary gas (20 and 10 arbitrary units), while argon was used as the collision gas (1.5 arbitrary units). Two precursor product ion transitions for each analyte and internal standards were used for quantification. The transitions selected for MSMS analysis and retention times are shown in supplementary materials (Table S3). Data were acquired and processed using Excalibur software.

The limits of detection (LOD) and quantification (LOQ) represent three and ten times the ratio of the average noise height on either side of a known amount of a compound's peak to the peak height, respectively. The objective was to determine the minimum peak heights that can be used to distinguish a compound's peak from the noise on either side of the peak. The LOD and LOQ determined for each sample type are presented in Table S3. LODs varied from 0.001 to 0.012 pg μL $^{-1}$ and LOQs from 0.005 to 0.041 pg μL $^{-1}$.

3. Results

No neonics were detected in blackbirds. For the other species, all 5 neonics searched, namely, clothianidin, dinotefuran, nitenpyram, thiacloprid, and thiamethoxam were detected. A summary of the concentrations and occurrence of these neonics in our sampled bird species is provided in Table 1, and detailed neonicotinoid distribution among each individual is provided in Fig. 2. Clothianidin was found in all species (except blackbirds), with average concentrations ranging from 0.05 pg μL^{-1} in common nightingales to 951.60 pg μL^{-1} in Montagu's harriers. The number of individuals that exhibited concentrations higher than the LOQ ranged from ~5% among Montagu's harriers up to ~26% among grey partridges sampled during the 2021–2022 winter. No clothianidin was detected in grey partridges sampled in the 2020–2021 winter. Thiacloprid was detected in all cirl buntings and common nightingales but always at concentrations below the LOQ. It was detected in one Montagu's harrier chick (89.58 pg μL^{-1}) and one grey partridge (0.07 pg μL^{-1}) in the 2020–2021 winter. Thiamethoxam was detected in both passerine species and in grey partridges from both winters, with concentrations ranging from 0.06 pg μL^{-1} in a common nightingale to 23.73 pg μL^{-1} in a grey partridge from the 2020–2021 winter. The number of individuals that exhibited concentrations higher than the LOD ranged from ~4% among common nightingales up to 12% among cirl buntings. Dinotefuran and nitenpyram were only detected in grey partridges: in the first winter (2020–2021), ~13% and ~87% of the individuals exhibited dinotefuran and nitenpyram concentrations higher than the LOD, with the average concentrations being 6.20 and 23.10 pg μL^{-1}, respectively. In the second winter (2021–2022), dinotefuran and nitenpyram were detected with concentrations higher than the LOD in ~32 and ~94% of the individuals, respectively, with the

Table 1

Summary of the mean concentrations [c] with standard deviations (SD) and range in pg µL^{-1} with their sample size (n) obtained by LC-MS/MS above the limits of detection (LOD) for each species. The sample size for each species is also provided (N). The percentage of samples above the LOD (n/N) is given in brackets (rounded to the nearest percent). Blackbirds are not referenced as no neonic was detected in the sampled individuals.

		Clothianidin	Dinotefuran	Nitenpyram	Thiacloprid	Thiamethoxam
Cirl buntings (N = 34)						
	n	5 (*15%*)	0	0	34 (*100%*)	4 (*12%*)
	[c] ± SD	2.28 ± 1.55	–	–	LOD < [c] < LOQ	2.59 ± 1.37
	range	1.04–4.87	–	–	–	1.61–4.56
Common nightingales (N = 25)						
	n	2 (*8%*)	0	0	25 (*100%*)	1 (*4%*)
	[c] ± SD	0.05 ± 0.06	–	–	LOD < [c] < LOQ	0.06
	range	0.009–0.093	–	–	–	–
Grey partridges (N = 54)						
Winter 2020/2021 (N = 23)	n	0	3 (*13%*)	20 (*87%*)	1 (*4%*)	1 (*4%*)
	[c] ± SD	–	6.20 ± 3.62	23.10 ± 11.33	0.07	23.73
	range	–	2.14–9.10	1.24–41.03	–	–
Winter 2021/2022 (N = 31)	n	8 (*26%*)	10 (*32%*)	29 (*94%*)	0	3 (*10%*)
	[c] ± SD	5.26 ± 4.10	7.85 ± 4.07	18.29 ± 10.84	–	1.64 ± 0.46
	range	1.92–14.26	3.32–16.61	1.78–43.53	–	1.23–2.14
Montagu's harriers (N = 55)						
	n	3 (*5%*)	0	0	1 (*2%*)	0
	[c] ± SD	951.60 ± 1299.21	–	–	89.58	–
	range	194.48–2451.78	–	–	–	–

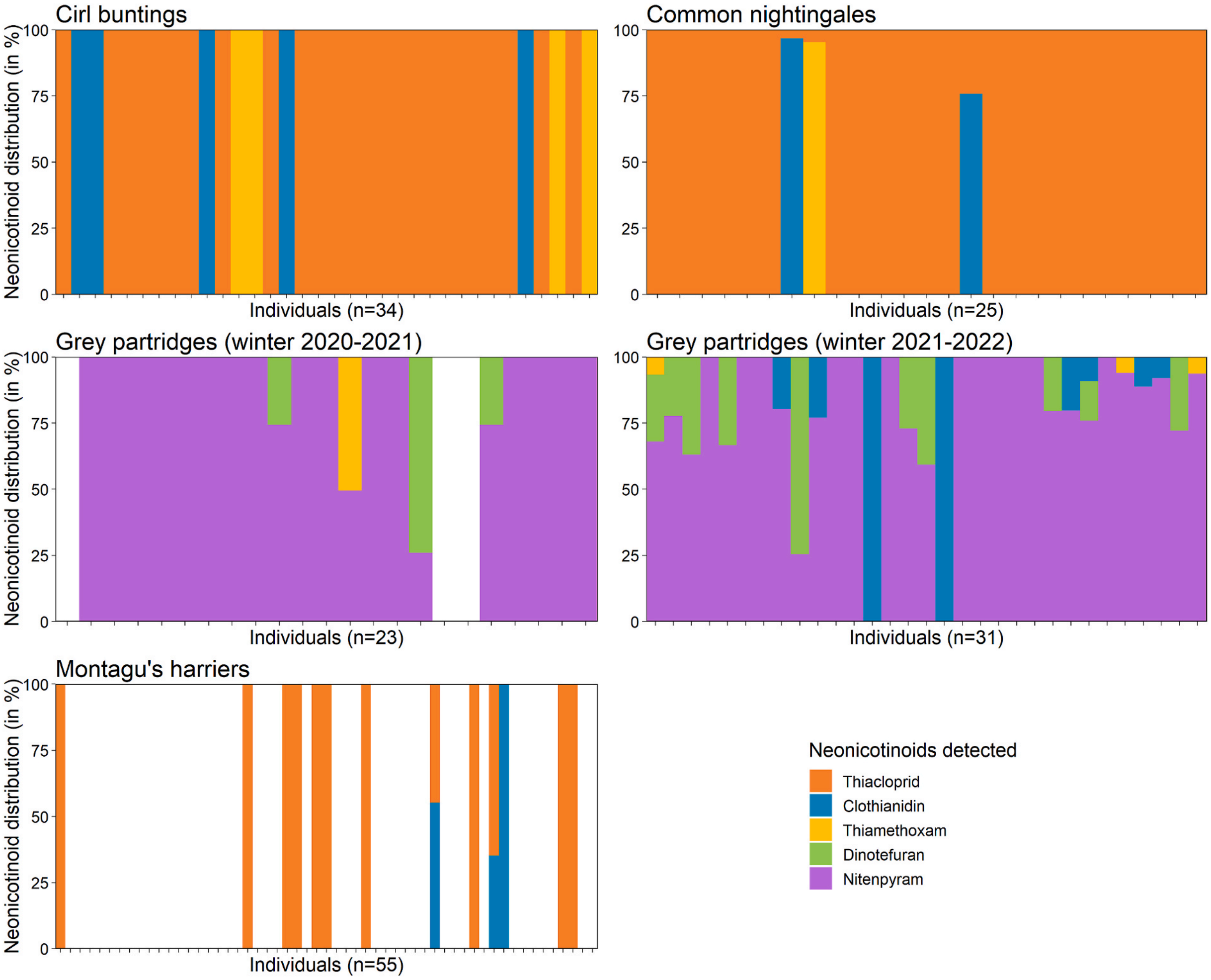

Fig. 2. Wild farmland birds' exposure to 5 neonicotinoids. Distribution of the different neonicotinoid molecules for each individual of each species are represented as one stacked bar. Distribution in percent was obtained from raw concentrations of each molecule in birds blood sampled.

average concentrations being 7.85 and 18.29 pg μL^{-1}, respectively.

4. Discussion

In the last few decades, neonics have attracted considerable interest (Klingelhöfer et al., 2022), especially because of the rising concern about their effects on non-target species such as honeybees (Wood and Goulson, 2017) and humans (Cimino et al., 2017; Han et al., 2018). Because of their chemical properties such as their half-life in the soil (DT_{50}), solvability, and leaching potential, and because of their extensive use for agricultural purposes, the assessment of their presence in farmlands is imperative (Thompson et al., 2020). Considering that no neonics were used on plants since 2018 in France and that the analytical method used here provided good sensitivity results (Rodrigues et al., 2023), we expected to find no or very low concentrations in bird blood samples as this matrix reflects short-term exposure (Espín et al., 2016). However, in the present study, we not only detected five out of seven neonics, three of which have been banned since 2018 in France (clothianidin, thiacloprid, and thiamethoxam) and two others (dinotefuran and nitenpyram) that are supposedly being used only for treating domestic pets (Table S2), but in some cases, at rather high concentrations. The remaining two neonics (imidacloprid and acetamiprid) are not discussed here as these compounds were not detectable with the multiresidue method used in this study. However, knowing the historical background of the study area and results from studies in the same area before neonics ban (see section 2.1), if titration had been possible, we would have expected to find large amounts of imidacloprid in birds' blood and probably no or very low concentrations of acetamiprid.

4.1. Monitoring neonics in wild birds

Neonics were increasingly studied during the last decade and several recent studies have focused on quantifying the level of exposure in wild birds. However, these studies differ from our work in several aspects. First, in these studies, several biological matrices—blood, carcasses, eggs, faecal pellets and cloacal fluids, feathers, liver, and/or gizzard—were considered (see Table S1 for detailed references). Correlations between neonics quantifications from different matrices can be found but are not systematic (reviewed in Espín et al., 2016). Indeed, pesticides are distributed in biological tissues with different temporal patterns. For instance, pesticide molecules are integrated into feathers during the moulting period (Humann-Guilleminot et al., 2022). The distribution of such neonic molecules in internal tissues such as the liver also depends on the chemical nature of the molecules and can be degraded if samples are taken on carcasses (Espín et al., 2016, and references therein). Then, a time lag might be observed between the exposure and the detection depending on the matrices used (Lennon et al., 2020b). Therefore, the results obtained from different matrices cannot be directly compared.

Our results can thus be reasonably compared to those of five previous studies that tested for neonics in whole blood (or plasma) samples from wild birds (Taliansky-Chamudis et al., 2017; Byholm et al., 2018; Hao et al., 2018; Lennon et al., 2020b; and Humann-Guilleminot et al., 2021; Table S4). For clothianidin, our concentration range was similar to those found by Lennon et al. (2020b), although the maximum value was lower: they found concentrations ranging from 0.5 (dunnocks) to 69, 300 pg μL^{-1} (yellowhammers), whereas in our study, the values were 0.009 (common nightingale) to 2451.78 pg μL^{-1} (Montagu's harrier chick). The maximum clothianidin concentration found by Humann-Guilleminot et al. (2021) was 0.34 pg μL^{-1} in alpine swifts, which is similar to the lower values in the concentration range we obtained. Thiacloprid concentrations in all cirl buntings and common nightingales were below the LOQ, but concentrations in the grey partridges and the Montagu's harrier chicks were far higher than those found in honey buzzards by Byholm et al. (2018) and in white-crowned sparrows by Hao et al. (2018). They ranged from 0.012 to 0.031 and 0.0025 to 0.0031 pg μL^{-1} for honey buzzards and white-crowned sparrows, respectively, and from 0.073 to 89.58 pg μL^{-1} in our study (Table S4). This result suggests that the grey partridges and the Montagu's harrier chicks were exposed to high, or recent quantities of thiacloprid (see section 4.2 for further details). For thiamethoxam, the concentrations we found were above those obtained by Hao et al. (2018): they ranged from 0.06 (common nightingales) to 23.73 (grey partridges) pg μL^{-1}, whereas Hao et al. reported 0.0051 to 0.0337 pg μL^{-1}. Taliansky-Chamudis et al. (2017) detected imidacloprid in one Eurasian eagle owl (*Bubo bubo*) chick among the 30 sampled, but did not detect any other neonics, so comparisons with our results are not possible. The difference in the results despite all studies using blood samples may be because some of the studies considered whole blood samples (Taliansky-Chamudis et al., 2017; Byholm et al., 2018; our study), while others used only plasma (Hao et al., 2018; Lennon et al., 2020b; Humann-Guilleminot et al., 2021), inducing differences in the detectability of some molecules whose levels vary according to their water/lipid solubility, and affinity to different proteins (Rodrigues et al., 2023; Zhang et al., 2023). Moreover, all these studies fundamentally differ in their sensitivity as they rely on their own developed chemical analysis methods [although they are all derived from the same method, i.e., Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS); Anastassiades et al., 2003]. If we consider clothianidin for instance, LOD and LOQ in our study (0.01 and 0.04 pg μL^{-1}, respectively; Table S3) were 5 (LOQ) and 15 (LOD) times lower, respectively, than those (0.15 and 0.21 pg μL^{-1}, respectively) of Lennon et al. (2020b), while LOQ was similar to that (0.05 pg μL^{-1}) reported by Humann-Guilleminot et al. (2021). This may induce a difference in the number of neonic positive samples between studies, hence differences in average concentrations as well. For instance, applying the LOD of clothianidin from the study of Lennon et al. (2020b) would have led to non-detection of positive samples in common nightingales while we found 8% of individuals with concentrations above our LOD. It is not therefore easy to compare the results provided by the authors, especially when not all statistical values, such as the percentage of neonic detection among individuals or the mean concentration with its standard deviation, were obtained on the same basis (Table S4). As the use of the same methodology in all studies does not seem to be feasible, researchers should systematically report these values to allow direct comparisons.

4.2. Exposure of wild fauna to neonics

Previous studies reported the limitations of feather and internal tissue samples for determining timing of exposure to contaminants, so blood is considered most suitable for determining recent exposure (Espín et al., 2016; Lennon et al., 2020b). Neonics are supposedly "rapidly" excreted (in hummingbirds, English et al., 2021) and cleared from blood (24 h in quails), according to data regarding imidacloprid (Bean et al., 2019) and thiamethoxam (Pan et al., 2022); however, extrapolation to the behaviour of other neonics may not be reliable. Based on blood samples used for this study we cannot estimate long-term exposure, therefore, possible exposure of the migratory species at their wintering areas may be disregarded. However, thiamethoxam/clothianidin were found in grey partridges' eggs in another study (Bro et al., 2016), so in the case of the Montagu's harrier chicks, we cannot exclude the possibility of maternal transfer of neonics, i.e., from the mother to the eggs. In other words, all tested positive individuals in the present study were probably recently exposed to neonics. This is important because the birds were sampled three and four years after EU banned neonics for PPP, and no derogation is known in our study area conversely to other regions in France. Nonetheless, we found three out of the five PPP neonics in our samples, with concentrations similar to those in birds sampled elsewhere before the ban (Byholm et al., 2018). Taken together, our results strongly suggest that clothianidin, thiacloprid, and thiamethoxam are still present in farmlands and raise questions about the mode of exposure to birds. Thiamethoxam is metabolized into clothianidin in animals, plants, and soil (Nauen et al., 2003, review in Simon-Delso et al., 2015; Pan et al., 2022) potentially explaining why it is more commonly present than thiamethoxam. These neonics' DT_{50} ranged from few days for thiacloprid up to several years for clothianidin (Table S2) in the soil, meaning that they can still be incorporated into the diet of several detritivores such as earthworms (Pelosi et al., 2022). If so, at least some blackbirds, which are omnivorous and feed on different invertebrate species during the breeding season, including earthworms which are known to bioaccumulate pesticides (Pelosi et al., 2022, 2021), should have tested positive, but were not. This surprising result could have been first seen as a technical failure; however, other pesticide molecules than neonics were detected in blackbirds' blood, so that an analysis problem can be discarded. The absence of neonics in blackbirds' blood might be the consequence of several processes. One of them could be a higher detoxification capacity of blackbirds, mediated by their ability at monopolizing high amounts of carotenoids, which are antioxidants, involved in sexual selection and known for their role in reducing oxidative stress (Møller et al., 2000, see Moreau et al., 2022a). Further analyses would be needed to explore this explanation. Granivorous birds, including grey partridges (as they were caught during the winter), cirl buntings, and common nightingales, which are omnivorous (mostly insectivorous during reproduction), tested positive. This suggests that the mode of exposure may include contaminated seeds from previous treated crops (Wintermantel et al., 2018) or organisms feeding on contaminated seeds. The higher concentrations found in Montagu's harriers, which are apex predators (mostly preying upon common voles and orthopterans), might be a result of biomagnification, i.e., the accumulation of toxic neonics along the trophic chain (Badry et al., 2020). In fact, clothianidin and thiacloprid, two compounds quantified in Montagu's harriers' blood, were found in hairs of small mammals from the area, reinforcing that view (Fritsch et al., 2022). Further investigations would be needed, and one way to do so would be using chicks' food pellets. These differences among species may also be attributable to the choices of individuals regarding their habitat and feeding resources for avoiding contaminated sources, as has been highlighted previously (McKay et al., 1999; Ruuskanen et al., 2020; Addy-Orduna et al., 2022). Another explanation relies on the detoxification processes in wild birds that are still poorly investigated and deserved further investigations (Moreau et al., 2022a). Indeed, some individuals could be more efficient than others at protecting themselves against toxic substances (Arnold et al., 2015).

Another important issue observed in our results is the high prevalence of dinotefuran and nitenpyram in grey partridges, although wildlife fauna is not supposed to be exposed to these as they are used only for pets and not farm animals, and consequently not for outdoor use in Europe, including France (Table S2). Even if grey partridges caught in this study area may originate mainly from captive breeding stocks (released for hunting purpose), the six registered veterinary medicines containing dinotefuran and nitempyram neonics that are authorized for use in France are not intended for treating any bird species or farm animal (http://www.ircp.anmv.anses.fr/index.aspx). Imidacloprid is included in 36 different speciality medications, making this neonic potentially more common even if not detectable here (see, for example, Perkins et al., 2021). Neonics are either used for topical applications on the skin (dinotefuran) or *per os* (nitenpyram) to treat flea infestations in cats and dogs typically; further, nitenpyram is expected to be eliminated within 48 h (Jeschke and Nauen, 2005; Rust, 2017). In Europe, veterinary regulatory processes are governed by the European Medicines Agency, which states that products for non-food animals are not supposed to be of major environmental concerns since these animals are treated individually with low concentrations of active neonics (CVMP/VICH, 2000). To our knowledge, these two neonics, investigated in few studies (Table S1), have only been detected in one sample of hummingbirds' feather rinsate (i.e., not within the organism; Graves et al., 2019), suggesting contact and not ingestion. In the present study, the substantial concentrations of dinotefuran and nitenpyram found in grey partridges but not in any other species may indicate possible exposure during their stay in farms before release. This implies either an illegal use of these substances in farms, as they are not included in veterinary medicines for farm animals, or an unintentional contact of partridges through multiple potential pathways. One of these could be direct contact and/or ingestion of farmer's dogs or cats' urine, or indirect through contaminated drinking water. However, neonics being presumably rapidly excreted and blood reflecting short-term exposure, individuals sampled here, if contaminated in farms before release, should not test positive. Thus, we cannot exclude in our case that partridges once in the wild could have directly ingested contaminated water with cat and/or dog urine and/or after pet baths (Teerlink et al., 2017; Diepens et al., 2023). Besides, partridges that commonly use field margins contrary to the other species, where pets walk and urinate, may have ingested these neonics while preening as the external surface of feathers are often contaminated and might even accumulate compounds (Pacyna-Kuchta, 2023). Indeed, there is growing evidence of veterinary products' transfer to the environment from dogs' hair and urine, and of secondary transfer to wildlife through nesting material or contaminated water (Diepens et al., 2023). Even though we cannot ascertain by which route the partridges were exposed to these neonics, our results indicate in line with Diepens et al. (2023), the urgent need for monitoring all pesticide or medicine compounds, irrespective of their intended use. Although treating domestic animals against ectoparasites might be of sanitary importance for public health (human and animal), these treatments may have the same adverse effects on wildlife as PPP (reviewed in Moreau et al., 2022a).

It should be noted that the interpretations given to the results provided in the present study are only assumptions and that the origin of birds' contamination remain unknown. Ongoing studies on neonics levels in soils and invertebrates of the study area since 2018 should help to provide further clarifications on birds' contamination pathways. Moreover, the method of multiresidue analysis used here could be improved to allow the detection of all neonics, including imidacloprid and acetamiprid, especially as imidacloprid was extensively applied in the crops of the study area and is still highly used in veterinary medicine.

4.3. *Conclusions*

Our results highlight several problems with the use of a class of chemicals that are among the most used worldwide. First, banning neonics for outdoor use does not prevent the exposure of wildlife fauna to them, at least a few years after ban. Although illegal use cannot be disregarded, it cannot explain on its own their ubiquity in wild birds from a wide ecological range, caught at the scale of the study site. This is an important element to consider in countries where neonics are still massively applied, considering their impact on both animals and humans (reviewed in Moreau et al., 2022a). Second, the detection of neonics used for domestic animals in wildlife fauna raises questions regarding the manner in which risk assessment for such applications is performed (Perkins et al., 2021; Diepens et al., 2023). For instance, to our knowledge, few studies have investigated the effects of dinotefuran and nitenpyram on wildlife fauna (Wang et al., 2018). Although clothianidin, dinotefuran, nitenpyram, and thiamethoxam are considered to be of low toxicity to birds, as indicated by their acute oral LD_{50} (Table S2), thiacloprid, which was also found to be quite ubiquitous, is highly toxic to birds (Table S2). However, LD_{50} is indicative of acute lethal toxicity under laboratory conditions for model species and is not necessarily an appropriate estimate of sublethal effects, as shown in the numerous studies that investigated the adverse effects of neonics on birds (Moreau et al., 2022a). Considering that very low residual levels of pesticides may have considerable sublethal effects on birds' reproduction (see Moreau et al., 2021, for example), the consequences of these exposures on bird populations as well as on human health (*One Health* concept) should be carefully considered.

Ethical statement

This study was conducted conforming the French guidelines for the ethical use of animals in research (APAFIS#18557-2019010822312199v2, APAFIS#9465e201703101551625). We are grateful to the Nouvelle-Aquitaine Regional Agency of the Environment, Development and Housing for the official authorizations for the capture of passerines birds (DREAL/2019D/2323). Because Montagu's harrier is a protected raptor species, its handling was allowed and licensed by the Centre de Recherches sur la Biologie des Populations d'Oiseaux – Museum National d'Histoire Naturelle (license #1308 for Montagu's harriers).

Credit author statement

EF, AG, AR, MM, VB, JM and KM conceived the ideas and designed the methodology; AG, JM and KM collected the data; EF, AR, MM and KM analysed the data; EF, AG, VB, JM and KM led the writing of the paper. All the authors contributed critically to the drafts and gave final approval for publication.

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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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Appendix A. Supplementary data

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Corrigendum

Corrigendum to “Neonicotinoids: Still present in farmland birds despite their ban, Chemosphere, **321**, April 2023, 138091”

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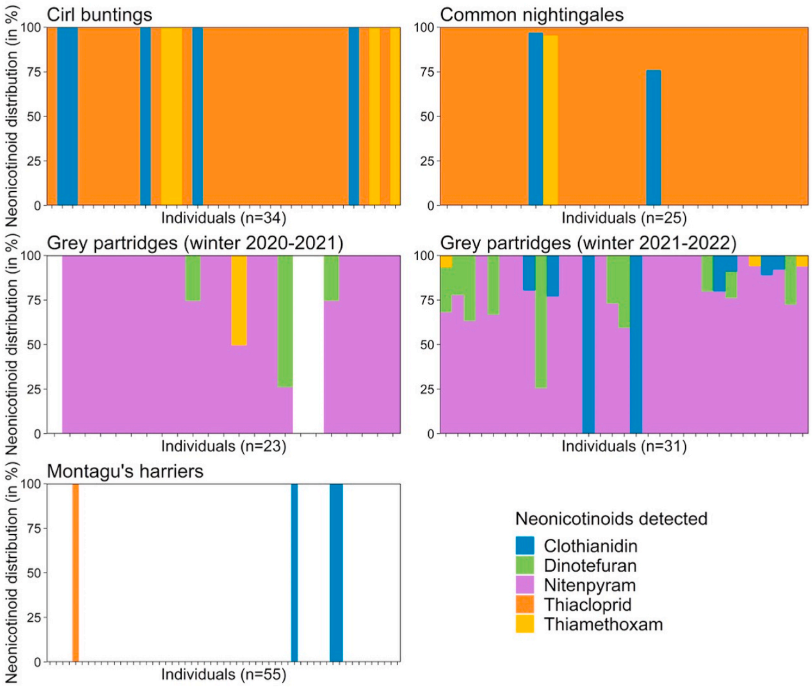
The authors regret a mistake on the **Figure 2** concerning Montagu’s harrier plot. Data used for plotting Montagu’s harrier contamination did not correspond to the data used for summary statistics of the results, due to a confusion in the data column used in R software. Consequently, the number of individuals with thiacloprid contamination appeared to be 12 in the plot while there is actually a single individual with a determined concentration of thiacloprid (given in **Table 1**).

Here is the correct **Figure 2**.

The authors would like to apologize for any inconvenience caused.



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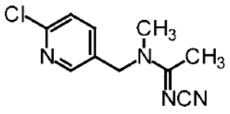
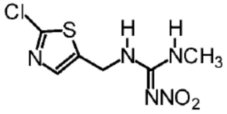
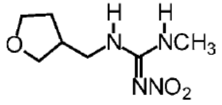
Supplementary material

Table S1. Examples of studies that used different matrices to quantify neonicotinoid insecticides in free-living birds from different trophic levels. Molecules quantified are ACE = acetamiprid, CLO = clothianidin, DINO = dinotefuran, IMI = imidacloprid, NIT = nitenpyram, THIA = thiacloprid, and TMX = thiamethoxam.

References	**Species**	**Feeding behavior**	**Matrix used**	**Molecules detected**						
				ACE	CLO	DINO	IMI	NIT	THIA	TMX
This study	*Circus pygargus*	Carnivorous	Blood		✓	✓		✓	✓	✓
	Emberiza cirlus	Omnivorous	Blood		✓	✓		✓	✓	✓
	Luscinia megarhynchos	Omnivorous	Blood		✓	✓		✓	✓	✓
	Perdix perdix	Granivorous	Blood		✓	✓		✓	✓	✓
Roy & Chen, 2023	*Tympanuchus cupido*	Omnivorous	Feces, Liver, Carcasses	✓	✓	✓	✓	✓	✓	✓
	Tympanuchus phasianellus	Omnivorous	Feces, Liver, Carcasses	✓	✓	✓	✓	✓	✓	✓
Distefano et al., 2022	*Ichthyaetus melanocephalus*	Piscivorous	Feathers	✓	✓		✓		✓	✓
	Thalasseus sandvicensis	Piscivorous	Feathers	✓	✓		✓		✓	✓
Graves et al., 2022	*Agelaius tricolor*	Granivorous	Carcasses	✓	✓	✓	✓	✓	✓	✓
Badry et al., 2021	*Accipiter gentilis*	Carnivorous	Liver		✓		✓		✓	
	Accipiter nisus	Carnivorous	Liver		✓		✓		✓	
	Haliaeetus albicilla	Carnivorous	Liver		✓		✓		✓	
	Milvus milvus	Carnivorous	Liver		✓		✓		✓	
	Pandion haliaetus	Piscivorous	Liver		✓		✓		✓	
Humann-Guilleminot et al., 2021	*Tachymarptis melba*	Insectivorous	Food boluses, Plasma	✓	✓		✓		✓	✓
	Tyto alba	Apex predator	Feathers	✓	✓		✓		✓	✓
Bishop et al., 2020	*Archilochus alexandri*	Nectarivorous	Cloacal fluid	✓	✓		✓		✓	✓

	Archilocus colubris	Nectarivorous	Cloacal fluid	✓	✓		✓		✓	✓
	Calypte anna	Nectarivorous	Cloacal fluid	✓	✓		✓		✓	✓
	Selasphorus calliope	Nectarivorous	Cloacal fluid	✓	✓		✓		✓	✓
	Selasphorus rufus	Nectarivorous	Cloacal fluid	✓	✓		✓		✓	✓
Lennon et al., 2020	*Alectoris rufa*	Granivorous	Liver, Bp		✓					
	Columba palumbus	Herbivorous	Liver		✓					
	Perdix perdix	Granivorous	Liver, Bp		✓					
	Phasianus colchicus	Granivorous	Liver		✓					
Graves et al., 2019	*Archilochus alexandri*	Nectarivorous	Feathers, Carcasses	✓	✓	✓	✓	✓	✓	✓
	Calypte anna	Nectarivorous	Feathers, Carcasses	✓	✓	✓	✓	✓	✓	✓
Humann-Guilleminot et al., 2019	*Passer domesticus*	Granivorous	Feathers	✓	✓		✓		✓	✓
Bishop et al., 2018	*Calypte anna*	Nectarivorous	Fecal pellets, Cloacal fluid	✓	✓		✓		✓	✓
	Selasphorus rufus	Nectarivorous	Fecal pellets, Cloacal fluid	✓	✓		✓		✓	✓
Byholm et al., 2018	*Pernis apivorus*	Insectivorous	Blood	✓			✓		✓	
Ertl et al., 2018	*Colinus virginianus*	Herbivorous	Liver	✓	✓		✓			✓
Hao et al., 2018	*Zonotrichia leucophrys*	Granivorous	Plasma	✓	✓	✓	✓	✓	✓	✓
MacDonald et al., 2018	*Meleagris gallopavo*	Granivorous	Liver	✓	✓	✓	✓	✓	✓	✓
Taliansky-Chamudis et al., 2017	*Bubo bubo*	Apex predator	Blood	✓	✓	✓	✓	✓	✓	✓
Bro et al., 2016	*Perdix perdix*	Granivorous	Eggs	✓	✓	✓	✓	✓	✓	✓
Berny et al., 1999	Pigeons and partridges	Granivorous	Liver, Gizzard				✓			

Table S2. Main characteristics, legislative status, and toxicological profiles of neonicotinoids. These are nicotinic acetylcholine receptor (nAChR) competitive modulators, and the site of action is indicated in the column "Mode of Action". The year of prohibition of use in Europe and France is given for each molecule, and NA = "Not applicable" if the molecule is not considered as a phytopharmaceutical product (PPP) in Europe. The DT_{50} (Detection Time 50% = time to detect a 50% decrease in pesticide concentration) ranges provided show the minimum and maximum values from general literature or field studies and can vary greatly depending on the sources (for more details see Lewis et al., 2016). Model species corresponds to the organism for which the LD_{50} (Lethal Dose 50% = amount of pesticide killing 50% of the test animals) was obtained: *Colinus virginianus* (C.V.), *Coturnix japonica* (C.C.J.), *Anas platyrhynchos* (A.P.), and *Serinus canaria* (S.C.). Data were compiled from the Pesticide Properties DataBase (PPDB) of the University of Hertfordshire (http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm accessed date 15 November 2022; Lewis et al., 2016). Prohibition years in Europe and France were obtained from legislative texts (available at https://eur-lex.europa.eu/homepage.html and https://www.legifrance.gouv.fr/ accessed date 15 November 2022).

	Use	Main crops	Prohibition in Europe/France	Emergency authorization (France)	DT_{50} range (days)	Bird LD_{50} ($mg\cdot kg^{-1}$)	Model species	Mode of Action
Acetamiprid 	Insecticide	vegetables, fruit, cotton, ornamentals	No/2018	No	3	98	A.P.	Systemic with translaminar activity, stomach and contact action.
Clothianidin 	Insecticide	corn, rice, orchards	2018/2018	No	13.3–1386	430	C.V.	Translaminar and root systemic activity.
Dinotefuran 	Insecticide Veterinary substance	vegetables, fruit, turf, rice	NA	No	50–100	2000	C.C.J.	Systemic with contact and stomach action.

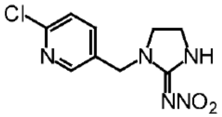
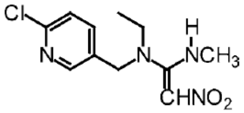
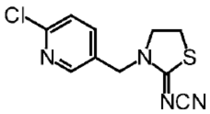
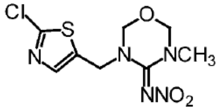
Imidacloprid 	Insecticide Veterinary substance	turf, rice, cereals, maize, potatoes, sugar beet	2018/2018	Yes	174	31	C.C.J.	Systemic with contact and stomach action.
Nitenpyram 	Insecticide Veterinary substance	glasshouse crops, rice	NA	No	1–15	1124	A.P.	Systemic with translaminar activity, stomach and contact action. No long-term activity.
Thiacloprid 	Insecticide	winterwheat, rape, seed, sugar beet, legumes	2020/2018	No	5.95–16.8	35	S.C.	Contact and stomach action with some systemic properties.
Thiamethoxam 	Insecticide	vegetables, turf, sod farms, landscape plants, ornamentals	2018/2018	Yes	7–72	576	A.P.	Broad spectrum, systemic with contact and stomach action.

Table S3. Limits of detection (LOD), limits of quantification (LOQ), multiple reactions monitoring (MRM) transitions, and retention time (rt) for each compound (two MRM transitions were monitored, product ion Q was used for quantification, and product ion q was used for qualification).

Compound	Precursor ion	Q	q	rt (min)	LOD (pg·µL^{-1})	LOQ (pg·µL^{-1})
Acetamiprid	223	99	126	6.24	-	-
Clothianidin	250	132	169	5.22	0.010	0.035
Dinotefuran	203	112	114	2.86	0.011	0.038
Imidacloprid	256	209	211	5.48	-	-
Nitenpyram	271	56	196	2.03	0.009	0.031
Thiacloprid	253	126	99	7.40	0.001	0.005
Thiamethoxam	292	181	211	4.35	0.012	0.042

Table S4. Comparison between studies that quantified neonicotinoids in blood of free-living birds. Molecules quantified are ACE = acetamiprid, CLO = clothianidin, DINO = dinotefuran, IMI = imidacloprid, NIT = nitenpyram, THIA = thiacloprid, and TMX = thiamethoxam. LOD = limits of detection and LOQ = limits of quantification; NA indicates the compounds' value was not provided by authors. Percentage of detection rounded to the nearest percent corresponds to the proportion of samples in which the neonicotinoid insecticide has been detected and is present in quantities greater than or equal to LOD or LOQ (specified in footnotes). [c] = mean or median concentration, SD = standard deviation, and IQR = interquartile range; by default mean ± SD are given (specified in footnotes when median ± IQR are given).

	LOD/LOQ in original measurement units	**LOD in pg·µL^{-1}**	**LOQ in pg·µL^{-1}**	**Species**	**% of detection**	**[c] ± SD or IQR (range)**		
						ng·mL^{-1}	**pg·mL^{-1}**	**pg·µL^{-1}**
This study[1]								
CLO	-	0.010	0.035	*Emberiza cirlus*	15	-	-	2.28 ± 1.55 (1.04-4.87)
				Common nightingale	8	-	-	0.05 ± 0.06 (0.009–0.093)
				Perdix perdix[4]	15	-	-	5.26 ± 4.10 (1.92-14.26)
				Circus pygargus	5	-	-	951.60 ± 1299.21 (194.48-2 451.78)
DINO	-	0.011	0.038	*Perdix perdix*[4]	24	-	-	7.47 ± 3.89 (2.14-16.61)
NIT	-	0.009	0.031	*Perdix perdix*[4]	91	-	-	20.25 ± 11.18 (1.24-43.53)

THIA	-	0.001	0.005	*Emberiza cirlus*	100	-	-	LOD < [c] < LOQ
				Common nightingale	100	-	-	LOD < [c] < LOQ
				Perdix perdix[4]	2	-	-	0.07
				Circus pygargus	2	-	-	89.58
TMX	-	0.012	0.042	*Emberiza cirlus*	12	-	-	2.59 ± 1.37 (1.61-4.56)
				Common nightingale	4	-	-	0.06
				Perdix perdix[4]	7	-	-	7.16 ± 11.05 (1.23-23.73)
Humann-Guilleminot et al., 2021[2]								
ACE	NA/0.01 $ng\cdot mL^{-1}$	NA	0.01	*Tachymarptis melba*	0	0 (0–0)	-	0 (0–0)
CLO	NA/0.05 $ng\cdot mL^{-1}$	NA	0.05		20	0.06 ± 0.11 (0–0.34)	-	0.06 ± 0.11 (0–0.34)
IMI	NA/0.05 $ng\cdot mL^{-1}$	NA	0.05		0	0 (0–0)	-	0 (0–0)
THIA	NA/0.01 $ng\cdot mL^{-1}$	NA	0.01		0	0 (0–0)	-	0 (0–0)
TMX	NA/0.03 $ng\cdot mL^{-1}$	NA	0.03		0	0 (0–0)	-	0 (0–0)

Lennon et al., 2020[1,3]								
CLO	0.15 ng·mL^{-1}/0.21 ng·mL^{-1}	0.15	0.21	*Alectoris rufa*	95	47.1 ± 382 (0.40–3200)	-	47.1 ± 382 (0.40–3200)
				Perdix perdix	83	1.80 ± 1.20 (0.60–3.00)	-	1.80 ± 1.20 (0.60–3.00)
Byholm et al., 2018[1]								
ACE	0.6-7.5 pg·mL^{-1}/NA	0.0006–0.0075	NA	*Pernis apivorus*	0	-	< LOD	< LOD
IMI	11-15 pg·mL^{-1}/39 pg·mL^{-1}	0.011–0.015	0.039		40	-	8.9	0.0089
THIA	1.6-2.0 pg·mL^{-1}/1.3-6.3 pg·mL^{-1}	0.0016–0.0020	0.0013–0.0063		70	-	15.43 ± 8.32 (12.00-31.00)	0.01543 ± 0.00832 (0.012–0.031)
Hao et al., 2018[1]								
ACE	3.6 pg·mL^{-1}/NA	0.0036	NA	*Zonotrichia leucophrys*	11	-	3.83 ± 0.10 (3.70-3.90)	0.00383 ± 0.0001 (0.0037–0.00390)
CLO	7.4 pg·mL^{-1}/NA	0.0074	NA		0	-	< LOD	< LOD
DINO	9.1 pg·mL^{-1}/NA	0.0091	NA		0	-	< LOD	< LOD
IMI	4.6 pg·mL^{-1}/NA	0.0046	NA		78	-	35.83 ± 42.88 (5.00-177.00)	0.03583 ± 0.04288 (0.005–0.177)
NIT	8.8 pg·mL^{-1}/NA	0.0088	NA		0	-	< LOD	< LOD

THIA	2.3 pg·mL^{-1}/NA	0.0023	NA		11	-	2.70 ± 0.27 (2.50-3.10)	0.0027 ± 0.00027 (0.0025–0.0031)
TMX	4.5 pg·mL^{-1}/NA	0.0045	NA		22	-	13.46 ± 9.58 (5.10-33.70)	0.01346 ± 0.00958 (0.0051–0.0337)
Taliansky-Chamudis et al., 2017[1]								
ACE	NA/2 ng·mL^{-1}	NA	2		0	< LOD	-	< LOD
CLO	NA/10 ng·mL^{-1}	NA	10		0	< LOD	-	< LOD
DINO	NA/10 ng·mL^{-1}	NA	10		0	< LOD	-	< LOD
IMI	NA/2 ng·mL^{-1}	NA	2	*Bubo bubo*	3	3.28	-	3.28
NIT	NA/10 ng·mL^{-1}	NA	10		0	< LOD	-	< LOD
THIA	NA/2 ng·mL^{-1}	NA	2		0	< LOD	-	< LOD
TMX	NA/10 ng·mL^{-1}	NA	10		0	< LOD	-	< LOD

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[1] Percentage of detection was obtained by including individuals with concentrations > LOD. [2] Percentage of detection was obtained by including individuals with values > LOQ. [3] Descriptive statistics given are median ± IQR. Descriptive statistics were obtained [4] for coupled data of *Perdix perdix* caught in 2020–2021 winter and 2021–2022 winter, [5] from online available dataset based on all sample sizes (including individuals with concentrations < LOD), or [6] based on data for sample size excluding individuals with concentrations < LOD.

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ARTICLE 7

APEX PREDATOR EXPOSURE TO PESTICIDES ASSOCIATED WITH GUT MICROBIOTA ALTERATIONS

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Apex predator exposure to pesticides associated with gut microbiota alterations

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Abstract

The gut microbiota, which ensures essential physiological functions for its host, can be disrupted by environmental perturbations. We show an assessment of gut microbiota disruption with exposure to several pesticides in a wild apex predator. We find alterations in key gut bacterial taxa abundances with possible impacts on host fitness, assuming a potential dysbiosis. This study sheds light on a significant perspective in our understanding of the potential harmful effects of pesticides on organisms, including humans, mediated by the gut microbiota.

Keywords: bacterial communities, cloaca, dysbiosis, farmland bird, microbiome, pollutants

1. Introduction

The gut microbiota is an essential modulator of host physiology and plays a key role in host health. Its importance is such that, in organisms, including humans, its disruption is associated with several pathologies, including diabetes, cancer, cardiovascular events, and neurodegenerative diseases (Hou et al., 2022). Among the possible sources of disruption, pesticides represent a major potential threat. Mostly ingested orally through water and food, pesticides are thought to alter the structure of the microbiota, the last barrier before entering the body. Until now, research has mainly focused on the effects of a single molecule on the composition of the microbiota, both in humans and in animal models, but mainly under laboratory conditions (Yuan et al., 2019). Wild birds are biosentinels of the ecosystem health and the organisms living here, especially apex predators such as raptors (Bodawatta et al., 2022; Handy et al., 2023). Indeed, these animals constitute relevant biological models by being at the top of the food web with potentially higher exposure due to biomagnification (Badry et al., 2020).

In the present study, we sampled Montagu's harrier (*Circus pygargus*) nestlings to examine the effects of pesticides on the composition and structure of the gut microbiota of an avian apex predator. In nestlings, gut colonisation occurs predominantly in the first days after hatching (Těšický et al., 2024) and is influenced by the environment and parental feeding (Diez-Méndez et al., 2023). Montagu's harriers are especially interesting in this context as they nest on the ground of cereal crops, the majority of which have been treated with pesticides before the breeding period, i.e., before May. The development of the nestlings' gut microbiota could, therefore, be influenced mostly by the local remanence from the soil and food provided by adults. Here, we describe a link between pesticide exposure measured in blood and alterations in the gut microbiota measured in cloacal samples controlling for nestling body condition and sex. We considered both the diversity of pesticides (i.e., the so-called cocktail effect) and the diversity of the gut microbiota.

2. Materials & Methods

2.1. Ethical consideration

All captures and experiments conformed to the French guidelines for the ethical use of animals in research (APAFIS#36725-2022041910139000 v6). Bird handling was allowed and licensed by the "Centre de Recherches sur la Biologie des Populations d'Oiseaux" (CRBPO; Museum National d'Histoire Naturelle; licence #1308).

2.2. Studied area and biological model

The study site "Zone Atelier Plaine & Val de Sèvre" is located in the Deux-Sèvres department (southwestern France) and covers 450 km². It is an area of intensive farming, whose landscape is dominated by winter cereal crops (i.e., on average 41.5% of the area between 2009 and 2016), with also crops including corn, sunflowers, and oilseed rape (Bretagnolle et al., 2018). Since 1994, Montagu's harriers have been monitored in the ZAPVS during the breeding period, after their return from migration mainly from Africa. This threatened, protected species nests on the ground, mainly in cereal crops, making chicks and adults directly subject to agricultural activity (Arroyo et al., 2002; Millon et al., 2002). In France, the populations of this bird are decreasing, probably due to the intensification of agricultural work in the last decades, making them of conservational priority but also limiting our sample size (Comolet-Tirman et al., 2015). On average, 2.05 fledglings per nest survive and fly out of the nest; this productivity depends mainly on available resources and especially their main prey (Salamolard et al., 2000). As a top predator, Montagu's harriers feed mostly on common voles (*Microtus arvalis*) and, in years of poor abundance, on orthopterans, making them prone to biomagnification and thus to high exposure to pesticides. Generally, males provide a food supply for incubating females and chicks and then females also participate during the chick-rearing period. Feeding areas in the ZAPVS are approximately 14 km² (Guixé and Arroyo, 2011).

2.3. Sampling

In 2022, professional ornithologists located and visited Montagu's harrier nests in the study area. Thirteen nests containing 22 chicks (11 males, 11 females) were visited before eggs hatched and subsequently every week. After the hatching, chicks were head-marked with a water-based coloured pen to allow individual identification, and 15 days later, they were banded with a unique coded aluminium ring provided by the Museum National d'Histoire Naturelle de Paris (France). They were also sexed according to their iris colour (i.e., brown for females, grey for males, Leroux and Bretagnolle, 1996). Then, when nestlings were 26 ± 2 days old, they were carefully handled in a short time to collect blood and microbiota samples frozen at −20°C and −80°C, respectively, before analyses. Blood samples were used to assess pesticide load by taking 50 µL from the brachial vein using a sterile needle and heparinised capillaries and then placed in Eppendorf tubes. The pesticide load in the blood was quantified as a measure of their recent exposure (Espín et al., 2016). Gut bacterial communities were sampled by gently inserting a sterile needleless syringe into the cloaca of each bird, injecting 1 mL of sterile phosphate-buffered saline, and then drawing it out again. Samples were immediately placed in sterile vials, kept in a coolbox in the field, and later stored at −80°C. While each part of the digestive tract harbours specific bacterial communities, microbial shifts incurred in the higher intestine of birds lead to concurrent shifts in cloacal communities, making cloacal sampling a reliable non-invasive technique to study inter-individual variability in gut communities (e.g., on various bird species, van Dongen et al., 2013; Teyssier et al., 2020).

2.4. Pesticide analysis

Pesticide analysis follows the method developed by Rodrigues et al. (2023). Using the whole blood samples, including red blood cells and plasma, the analytical method allowed the detection and quantification of 116 compounds of pesticide used in the ZAPVS and more globally in France, including active ingredients (fungicides, herbicides and insecticides) and synergists (which improve the action of pesticides) or safeners.

Each blood sample was thawed, weighed, mixed with 2 mL dichloromethane and ethyl acetate (1:1), and homogenised by vortexing for 1 min, followed by three rounds of sonication for 10 min each time. After this, centrifugation of samples was done for 5 min to pool supernatants before evaporating them under a fume hood until having a final volume of 500 µL. Then, the samples were concentrated and injected simultaneously into liquid chromatography (LC-MS/MS) or gas chromatography (GC-MS/MS, for more volatile compounds) for quantification. The LC-MS/MS analyses were carried out in MRM (Multiple Reaction Monitoring) mode with two mass transitions for each compound, one for quantification and one for qualification. The GC-MS/MS analyses were preceded by a thermal desorption step, and a MRM mode was also used for detection and quantification. The concentration of each pesticide detected in the blood of chicks was obtained in pg.mg^{-1}. Analytical quality assurance and control procedures are provided in the detailed description of the method by Rodrigues et al. (2023).

2.5. Gut microbiota analysis

The microbiota in cloacal samples was assessed using a metabarcoding approach with the bacterial 16S ribosomal RNA gene, present in all bacteria and archaea (Mizrahi-Man et al., 2013), at the Plateforme Genomique, Genopole Toulouse (France).

2.5.1. DNA extraction and amplification

Bacterial DNA was extracted following the method of DNeasy® PowerSoil® Pro Kit and the standard protocol designed for the purification of total DNA from Gram-positive bacteria (Qiagen, Venlo, Netherlands). Bacterial DNA was then amplified by PCR targeting the V3-V4 hypervariable regions of 16s rRNA in a room dedicated solely to metabarcoding procedures. The 20 µL PCR solution contained 2X Master Mix AmpliTaq Gold (Fisher), 0.008X BSA, 0.25 µM forward primer (GTGYCAGCMGCCGCGGTAA), 0.25 µM reverse primer (GGACTACNVGGGTWTCTAAT) and 2 µL DNA extract. First, a denaturation at 95 °C for 10 min took place, followed by 35 cycles of denaturation at different temperatures: 95 °C for 1 min, 50°C for 1 min, 72°C for 1 min 35 s and at 72°C for 10 min. Then, nine negative and nine positive controls were included in all amplifications to, respectively, avoid potential contamination and monitor the amplification's performance. Three PCR replicates were done for each sample, and the primers were tagged using a system of 36 octamers with at least five differences between them to obtain a unique tag combination for each PCR product.

2.5.2. Bioinformatics and data curation

Illumina sequencing data were processed and filtered using the OBITools package. The sequences were demultiplexed, dereplicated, and filtered according to their length (100 base pairs minimum) and their alignment quality (40% minimum, Taberlet et al., 2018). Sequences were then assigned to samples using the "ngsfilter" command, allowing 0 and 2 errors on the tags and primers, respectively, with identical sequences merged with the obiuniq command. Low-quality sequences, i.e., less than 1 read in the whole dataset, shorter than expected (<80 bp) or with ambiguous bases, were filtered out. After this cleaning, sequences were grouped into MOTUs (Molecular Taxonomic Operational Units), with sumaclust, according to a similarity threshold of 97%. For each MOTU, the most abundant sequence was considered its representative sequence, its abundance corresponding to the sum of the abundance of its members. The taxonomic assignment for each MOTU was carried out with the SILVAngs pipeline v.1.9.10 and the SILVA SSU database v138.1 (Quast et al., 2012). In addition to the obitools program, other artifactual sequences (PCR/sequencing errors, contaminant sequences, tag-jumps and dysfunctional PCRs) were identified using the R package MetabaR. MOTUs that did not belong to the targeted clade (i.e. Bacteria), with maximum abundance in the negative controls or with a similarity below 80% to the reference sequence were excluded. To standardise differences among samples, rarefaction was applied to 4300 reads.

2.6. Statistical analyses

We tested the concordance between the gut microbiota community structure and the concentration of pesticides measured on chicks' blood using a sparse partial least square discriminant analysis with blocks (block sPLS-DA) implemented in the 'mixOmics' package ('block.splsda' function, Rohart et al., 2017). Variables in each dataset were filtered to only keep the most frequent and abundant ones (occurring at least in ≥ 5 chicks). A model was constructed to test the level of congruence between the two datasets by verifying the correlations of eigenvalues of samples on components 1 and 2 of each dataset. Only strong correlations between MOTUs and pesticides with a |strength|> 0.6 were considered.

3. Results

In the blood of the 22 chicks, 36 pesticides and 139 MOTUs were detected, but we only kept those observed in at least five different chicks for analyses to ensure robustness. Thus, sPLS-DA included the 35 most prevalent MOTUs and 25 pesticides, which were found in the blood of at least five chicks and controlled for the chick's body condition as an essential component of their quality.

The eigenvalues for the first and second components of pesticide and microbiota matrices were highly correlated (Person's correlation test, 1st component: r = 0.82, P < 0.0001 and 2nd component: r = 0.75, P < 0.0001), indicating important underlying correlations between variables across each dataset (Figure 1). The strongest correlations (>|0.60|) involved eight different MOTUs (4 Actinobacteriota and 4 Proteobacteria, see Table 1) and 12 pesticides (two fungicides, seven herbicides, three insecticides), among which three were banned in Europe (acetochlor: 2013, chlorpyrifos: 2020, quinoxyfen: 2019) (Figure 2). Most MOTUs were positively correlated with pesticides except *Turicella sp.*, which was negatively correlated with acetochlor (Figure 2).

4. Discussion

The MOTUs were phylogenetically close to groups known for different roles that might be important to consider in the present case, with either beneficial or adverse effects on birds. These groups might be involved in bird metabolism, may induce pathogenicity, and/or be involved in bioremediation. For example, *Corynebacterium* sp. is commonly found in birds (Katsukawa et al., 2016; Pereira et al., 2024) and has been observed in higher abundance in migratory than in resident birds, which suggests a role in fat deposition metabolism (Zhang et al., 2021). Members of the Pseudomonadaceae family, such as *Alcaligenes* sp. or *Pseudomonas* sp., might be responsible for sanitary issues, such as respiratory disease in poultry for *A. faecalis* (Berkhoff et al., 1983) or be the vector of resistant genes that might be problematic in the current multiple drug-resistant crisis for *P. putida* (Li et al., 2021) (Table 1). In the meantime, these bacteria might also be helpful mediators of bird detoxification in being able to degrade some pesticide molecules such as chlorpyrifos (Ajaz et al., 2012) or, more widely, other organosphorous and neonicotinoid insecticides (review in Gilani et al., 2016 and Książek-Trela and Szpyrka, 2022). *Ralstonia* sp. (R. *syzygii* and *R. insidiosa*, Table 1) are commonly found in soils and plants and are also known to degrade organophosphorus pesticides such as diazinon (Wang and Liu, 2016).

Overall, MOTUs we detected were poorly known in birds and deserve further investigation to understand their effects as well as how pesticides may act on gut microbiota. Based only on the short and conserved 16S RNA, it is, however, difficult to formally identify bacteria that might be a major concern for nestling health. Nonetheless, our results are an important contribution in several respects. First, we show a significant correlation between the composition of the gut microbiota of an organism exposed to a cocktail of different pesticides found in its blood, suggesting that the processes linking microbiota and pesticide effects cannot be drawn from a single molecule nor a bacterial species, but rather has to be appreciated at the group level (for both pesticides and MOTUs). Second, our results suggest that pesticides may modulate the development of key bacterial taxa at early stages of the gut microbiota ontogeny, which may have later implications (due to priority effects) on the development of the adult microbiota and its health. Third, over and above the presence of banned molecules *per se* (see Fuentes et al., 2024), our results shed light on an overlooked collateral effect of pesticides, i.e., a general modification of gut bacterial assemblages. This can either lead to dysbiosis and the promotion of potential pathogens and/or the selection of bacteria that detoxify the organism, thus providing a form of adaptation to a polluted environment (at the expense of health or not; these two hypotheses are not exclusive).

Conclusion

Taken together, our study provides evidence for the pesticide influence on the microbiota structure in wildlife. It opens valuable perspectives at the cutting edge of our current knowledge for understanding the processes linking pesticide exposure and the construction of gut microbiota and further consequences on other physiological functions such as the immune system (Bodawatta et al., 2022; Ottinger et al., 2024).

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CRediT authorship contribution statement

Léa Bariod: Writing – original draft, Formal analysis, Conceptualization. **Elva Fuentes:** Writing – review & editing. **Maurice Millet:** Writing – review & editing, Formal analysis. **Joël White:** Writing – review & editing, Formal analysis, Conceptualization. **Samuel Jacquiod:** Writing – review & editing, Formal analysis. **Jérôme Moreau:** Writing – review & editing, Supervision, Conceptualization. **Karine Monceau:** Writing – review & editing, Supervision, Funding acquisition, Project administration, Conceptualization.

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Declaration of competing interest

The authors declare no competing interests.

Data Availability

Data will be made available on request.

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Table 1. Description of the 8 MOTUs correlated with pesticides, including results from NCBI Bast and putative identification.

Phylum Class Order Family	Genus	MOTUs	NCBI blast assignment (%)	Putative species
Actinobacteriota				
Actinobacteria				
Corynebacteriales				
Corynebacteriaceae	*Corynebacterium sp.*	OTU_048	100%	*C. jeddahense*
	Turicella sp.	OTU_040	100%	*T. otitidis* (syn. *Corynebacterium otitidis*)
Micrococcales				
Microbacteriaceae	*Leifsonia sp.*	OTU_059	100%	*L. xyli subsp. cynodontis, L. aquatica, L. shinshuensis, L. naganoensis*
	Micrococcus sp.	OTU_102	100%	*M. luteus* (3 strains), *M. aloeverae, M. endophyticus, M. yunnanensis, M. cohnii, M. antarcticus, M. flavus*
Proteobacteria				
Alphaproteobacteria				
Caulobacterales				
Caulobacteraceae	*Phenylobacterium sp.*	OTU_136	98.42-97.63%	*P. aquaticum, P. glaciei, P. koreense*
Gammaproteobacteria				
Burkholderiales				
Burkholderiaceae	*Ralstonia sp.*	OTU_003	100%	*R. syzygii* (4 strains), *R. insidiosa*
Pseudomonadales				
Pseudomonadaceae	*Alcaligenes sp.*	OTU_020	93.28%	*A. faecalis, A. aquatilis, A. parafaecalis, A. ammonioxydans*
	Pseudomonas sp.	OTU_099	100%	More than 10 different *Pseudomonas spp*. such as *P. argentiniensis, P. parafulva, P. flavescens, P. hunanensis, P. punonencis, P. cuatrocienegasensis, P. putida, P. fulva, P. faucium, P. promysalinigenes*

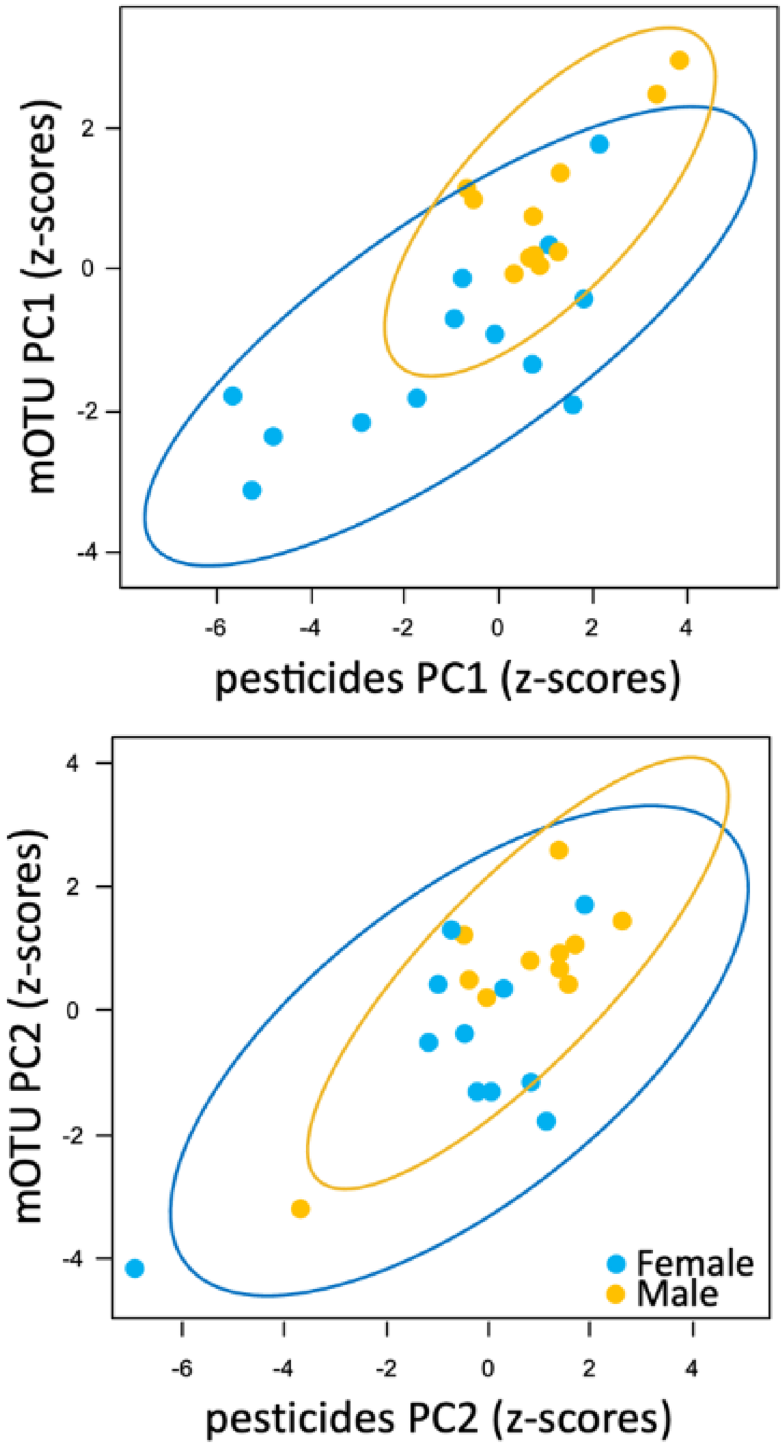

Figure 1. Pearson's correlation test between the axes of the principal component analyses (PCA) on MOTUs abundance and pesticide concentrations, a) correlation between the first axes of each PCA [PC1] and b) correlation between the second axes of each PCA [PC2].

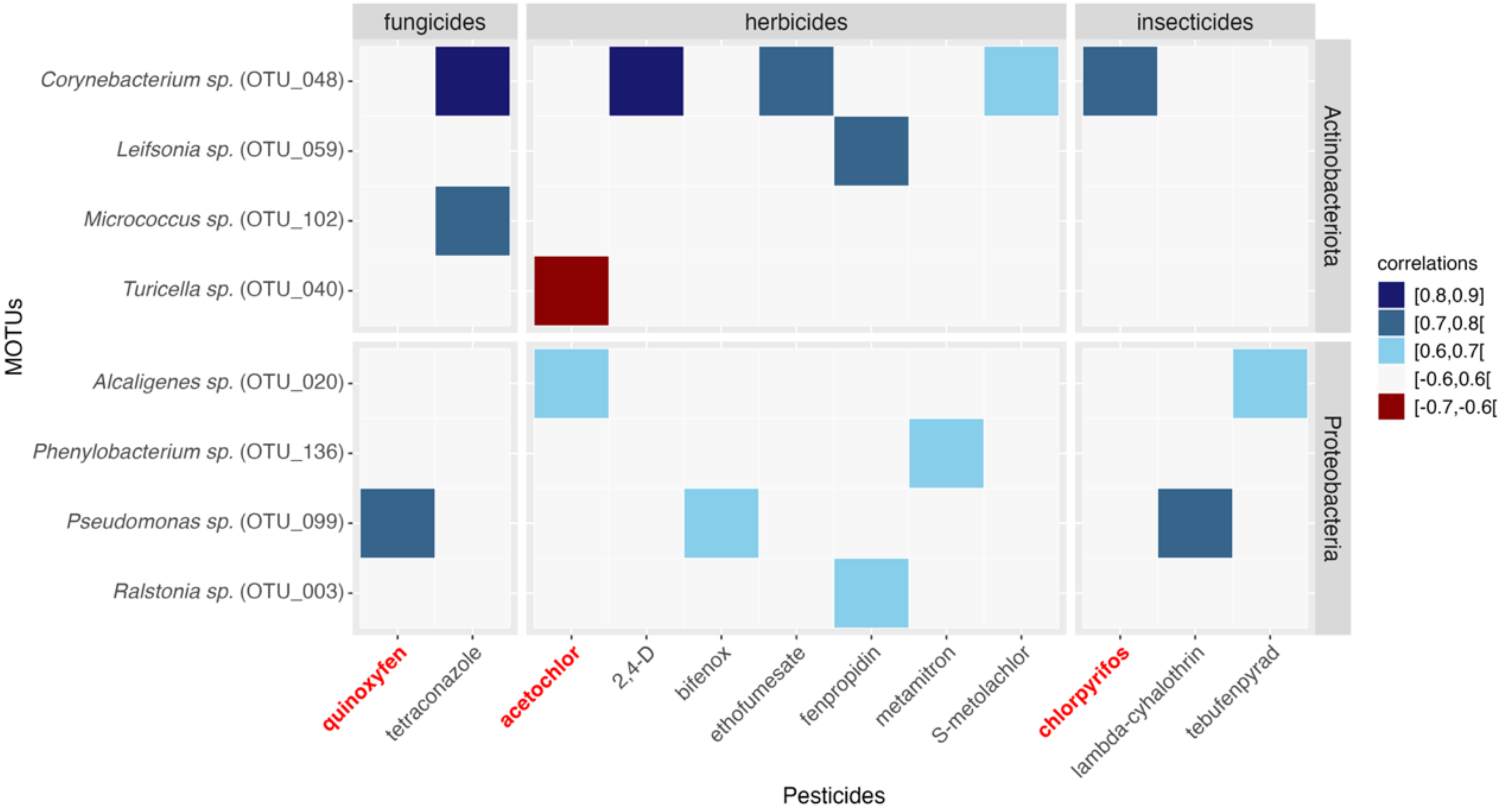

Figure 2. Heatmap representing correlation levels between pesticides and MOTUs. Pesticides in bold red are banned (see text for details).

ARTICLE 8

DIRECT AND INDIRECT EFFECTS OF PESTICIDE EXPOSURE ON FARMLAND RAPTOR GUT MICROBIOTA

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Direct and indirect effects of pesticide exposure on farmland raptor gut microbiota

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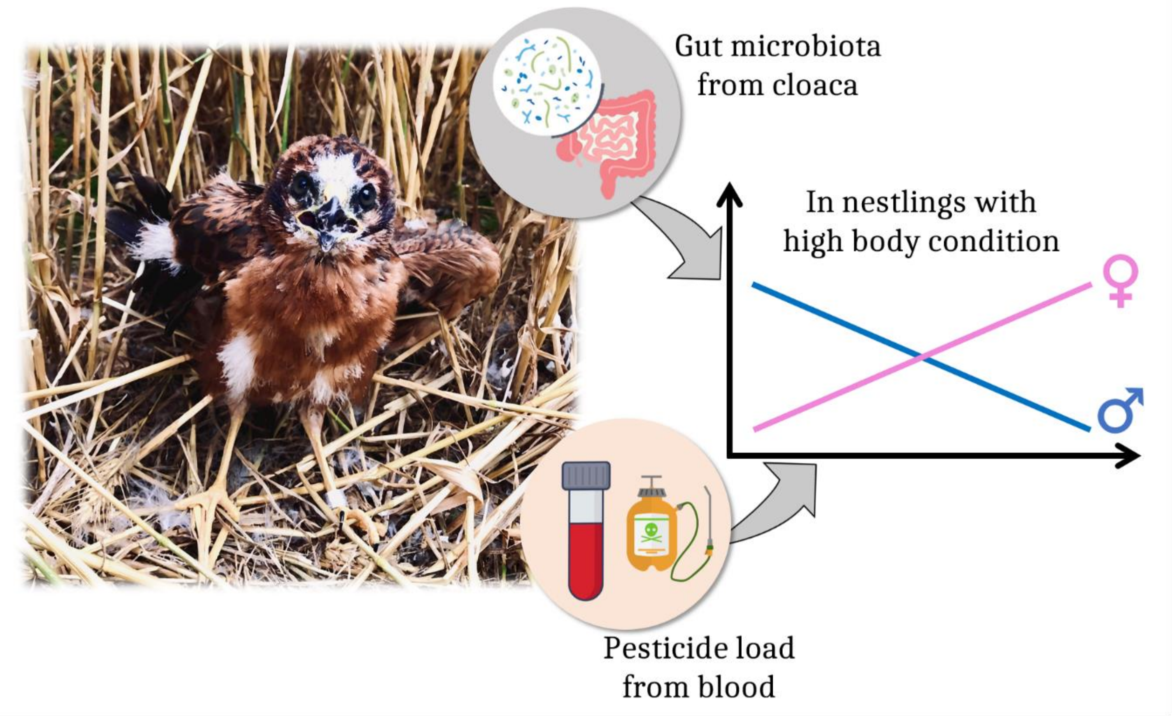
GRAPHICAL ABSTRACT



HIGHLIGHTS

- Pesticides may impact gut microbiota structure.
- 36 compounds of pesticides were detected in blood of Montagu’s harrier chicks.
- Microbial diversity shifts were related to pesticide load, sex and body condition.
- Proteobacteria were linked to lower pesticide load, Bacteroidota to higher levels.
- Farmland birds may serve as bio-sentinels in ecosystem health monitoring.

ABSTRACT

Recent studies in humans have shown that certain pesticides could affect the composition and functions of gut microbiota, an essential modulator of vertebrate physiology, leading to potential dysbiosis. However, this relationship remains largely unknown in wild birds despite the implications of pesticides in the current decline of farmland species. The present study sought to fill this gap by providing data on the association between pesticide concentrations in blood and gut microbiota characteristics in relation to individual traits in a farmland raptor, the Montagu's harrier (*Circus pygargus*). Results showed that females with higher body condition and higher pesticide load harboured a higher gut bacterial richness and diversity, while the relationship was opposite in males with higher body condition. Regarding taxonomic composition, Proteobacteria was the main phylum found in all nestlings. Differences in certain phylum and genus abundance according to pesticide load were found, with more Bacteroidota, *Leifsonia* and less *Bulkholderia* in nestlings with higher pesticide concentrations in their blood. Thus, this study highlights differences in microbiota and contamination by several pesticides according to the phenotypic characteristics of a wild raptor, and shows that farmland birds can represent relevant bio-sentinels for assessing the health/proper functioning of ecosystems (One Health approach).

Keywords: Chemicals, *Circus pygargus*, microbiome, microbiota, 16S amplicon sequencing

1. INTRODUCTION

The gut microbiota plays a central role in host metabolism and health (Clemente et al. 2012). It contributes to several physiological functions, such as energy harvest and storage (Den Besten et al. 2013), detoxification of trace metal elements (Liu et al. 2020), regulation of immunity and protection against pathogens (Gensollen et al. 2016; Bäumler and Sperandio 2016). However, this host-microbiota association can be disturbed by extrinsic factors such as antibiotic treatments, infections, changes in dietary habits, and other changes to the environment (Djekkoun et al. 2021). This disturbance, called dysbiosis, is often characterised by a reduction in beneficial microorganisms, concomitant to an increase in the number of potentially pathogenic microorganisms, leading to physiological disorders (e.g., digestive disorders, inflammation; Alberdi et al. 2016; Rinninella 2019). Among the disruptive factors, exposure to pesticides is likely to have a substantial impact on the structure of these microbial communities (Rosenfeld 2017; Lobionda et al. 2019). Indeed, pesticides are becoming a major health concern, especially in humans, because they can induce diverse pathologies such as metabolic diseases (Foley et al. 2018), dysregulation of the immune system (Mokarizadeh et al. 2015; Lee and Choi 2020), endocrine disruption (Frazier 2007) or gut dysbiosis leading to inflammatory damages and degenerative diseases (Banna et al. 2017; Lobionda et al. 2019). Current research on the toxicity of pesticides on gut microbiota is thus carried out in humans (Claus et al. 2016; Jin et al. 2017; Giambò et al. 2022), although recent studies in model species such as *Apis mellifera*, *Mus musculus*, and *Coturnix japonica* have demonstrated that increasing concentrations of some pesticides such as thiacloprid or trichlorfon in organisms decreased gut bacterial species diversity (Syromyatnikov et al. 2020; Giambò et al. 2021; Han et al. 2023). The disruption of the composition and, therefore, the function of this complex system can lead to significant metabolic imbalances, especially in glycolipid metabolism, as demonstrated in the gut microbiota of *Danio rerio* following exposure to carbendazim (Bao et al. 2020).

In birds, contaminant effects on gut microbial communities remain largely ignored, except for a few studies on experimental model species in controlled conditions (i.e., poultry, Shehata et al. 2013; Japanese quails *Coturnix japonica*, Crisol-Martínez et al. 2016; Ruuskanen et al. 2020; Han et al. 2023). However, captivity significantly alters the gut microbiota, likely due to the dietary, social and housing conditions, thus limiting extrapolations to wild species (Hird 2017). Wild birds remain understudied despite current concerns about their decline particularly that of farmland bird specialists due to the intensification of agriculture (Donal et al. 2001; Alexandratos and Bruinsma 2012). Indeed, across Europe, these avian populations are declining, and one of the causes behind this decline is the use of pesticides and their toxic effects (Rigal et al. 2023); however, empirical studies on the eco-evolutionary consequences of the long-term use of agrochemicals remain lacking (Ruuskanen et al. 2023). As they are pervasive in soil, water and air, pesticides are found in non-target organisms, even several years after banning (e.g., in mammals, Gibbons et al. 2015; Fritsch et al. 2022; in birds, Fuentes et al. 2023, 2024; in insects, Pelosi et al. 2021; Sánchez-Bayo 2021). In the literature, the privileged exposition pathway for birds is through food and/or water ingestion (Lopez-Antia et al. 2016).

Active chemical molecules pass through the digestive tract, are absorbed and then spread to various tissues *via* the bloodstream (Giambò et al. 2021). The gastrointestinal system, particularly the gut microbiota dwelling in this habitat, is the main interface between such synthetic chemical compounds and the organism's tissues. Given its involvement in processes linked to nutrient acquisition, the immune system and detoxification (Waite and Taylor 2015; Grond et al. 2018; Sun et al. 2022; Ottinger et al. 2024), it seems essential to integrate the gut microbiota into a global "One Health" approach to better understand the mechanisms linking pesticide exposure and avian population declines in rural areas (Handy et al. 2023). In this context, farmland birds might represent valuable biosentinels, as the link between pesticide exposure, the gut microbiota, immunity and health can be transposed to humans (One Health concept, One Health High-Level Expert et al. 2022; Handy et al. 2023).

The Montagu's harrier (*Circus pygargus*) is a migratory raptor species inhabiting agricultural lands, whose French populations have decreased by 10 to 30% over the last 30 years (Millon and Bretagnolle 2004; Comolet-Tirman et al. 2015), mainly due to agricultural intensification (Butet and Leroux 2001; Arroyo et al. 2002; Santangeli et al. 2015). This bird species nests on the ground in cereal crops where nestlings dwell for ca. 35 days (Arroyo et al. 2007). Consequently, they might be directly exposed to local pesticide contamination throughout their growth period, through direct spray on eggs, contact with chemicals on the soil and crop or especially *via* their diet (i.e., contaminated prey, see Fuentes et al. 2024). As a top predator, this species constitutes a relevant biological model, especially by being at the top of the food web with potentially higher exposure due to bioaccumulation/biomagnification (Badry et al. 2020). Given that microbial colonisation of bird guts occurs after hatching (Kohl 2012; Grond et al. 2018) and that developing organisms are generally more vulnerable to environmental stressors and pollutants (Ruuskanen et al. 2020), gut microbial communities in Montagu's harrier nestlings could, therefore substantially reflect the level of contamination of the habitat in which they are raised, and highlight the effects of these pollutants on the gut microbiota.

To our knowledge, this study is the first to investigate the relationships between the gut microbiota and pesticide load in a non-target organism, the Montagu's harrier, at the crucial stage of fledging. Contrary to other publications considering a single molecule (e.g., Shehata et al. 2013; Crisol-Martínez et al. 2016; Ruuskanen et al. 2020; Han et al. 2023), we capitalised on our ability to detect several pesticide molecules in bird blood (Rodrigues et al. 2023) to assess the degree of exposure of wild populations and to examine the link between this diversity of molecules (i.e., the so-called cocktail effect) and microbiota characteristics. We hypothesised gut bacterial communities to be modified in diversity and composition in relation to pesticide load in the blood (Syromyatnikov et al. 2020; Giambò et al. 2021). Given the deleterious effects of pesticides, we expected that nestlings with higher concentrations of pesticides detected in their blood would host gut bacterial communities with lower species diversity (Gois et al. 2023; White et al. 2023). Pesticide load may reduce intra-specific variability in gut composition if deterministic homogenising processes are at play (e.g., Cuesta-Maté et al. 2021) or, on the contrary, increase intra-specific variability if the stressor induces stochastic changes in microbiota composition (Zaneveld et al. 2017). Regarding specific taxa, we could expect a positive correlation between pesticide load and the relative abundance of phyla such as Bacteroides and Proteobacteria (e.g., *Coturnix japonica,* Crisol-Martínez et al. 2016; Han et al. 2023).

2. MATERIAL & METHODS

2.1. Study site and biological model

Data on Montagu's harrier nestlings were collected in the Long-Term Social-Ecological Research (LTSER) platform Zone Atelier Plaine & Val de Sèvre (ZAPVS), a study site covering 450 km^2, located in the *Deux-Sèvres* department (southwestern France; 46°11′N, 0°28′W). This area contains open landscapes where agricultural activity is intense: cereal crops are the predominant cultures (ca. 41.5% of the area between 2009 and 2016), followed by sunflowers and oilseed rape, with only a few urbanised areas and natural or semi-natural habitats (Bretagnolle et al. 2018).

In this area, Montagu's harrier population has been monitored since 1994 (Bretagnolle et al. 2018). This raptor nests on the ground, mainly in cereal crops and lays up to eight eggs (Arroyo et al. 1998), which are incubated by females for 29 days. Nestlings then remain for approximately 35 days in the nest before fledging (Arroyo et al. 2007). Female nestlings are, on average, larger than male nestlings in this species (Arroyo et al. 2002). In the study site, the mean productivity of this species is reported to be 2.05 fledglings per breeding attempt (Arroyo et al. 2004). The breeding success depends mainly on food availability (i.e., mainly voles and orthopterans if voles are scarce, Salamolard et al. 2000; Butet and Leroux 2001). These prey are mainly provided by males to females and nestlings by hunting further away from the nest, i.e., the home range of males observed in the study area being ~14 km^2 (Salamolard 1997). Females may also bring food items to nestlings by hunting in the vicinity of the nest (<1 km) before fledging (Wieringa et al. 2019).

2.2. Sampling

The reproduction of Montagu's harriers was monitored from late April to late July 2022. Twenty-two nestlings (11 males, 11 females) from 13 different nests were studied. The nests were visited twice before the eggs hatched and every week subsequently. After hatching, nestlings were head-marked with a water-based coloured pen to allow individual identification. At 15 days, they were ringed with a unique coded aluminium ring provided by Museum National d'Histoire Naturelle de Paris (France) and sexed according to their iris colour (i.e., brown for females, grey for males; Leroux and Bretagnolle 1996). Finally, when nestlings were 26 ± 2 days old, they were carefully handled in a short time frame (see different steps in Fuentes et al. 2023b) to weigh them with a Pesola 500 g spring scale (accuracy 5 g), to measure their left and right tarsus twice with a digital calliper (accuracy 0.1 mm), and to collect blood and microbiota samples. Based on tarsus length and body mass, we estimated the body condition of each nestling by using the scaled mass index (SMI) following Peig and Green (2009). In this study, males and females had an average (mean ± se) body condition of 294.22 ± 10.17 g and 320.63 ± 8.70 g, respectively. To assess pesticide load, 50 µL of blood was collected by puncturing the brachial vein using a sterile needle and heparinised capillaries and then placed in Eppendorf tubes. Gastrointestinal bacterial communities were sampled in the cloaca of nestlings. Cloacal bacterial communities have been shown to reflect bacterial communities in the higher intestine (Newbold et al. 2015) and have successfully been used to study the microbiota of wild birds, representing a reliable non-invasive technique to assess inter-individual variability in gut communities (White et al. 2010; Teyssier et al. 2018). Bacterial sampling was performed by washing the cloaca with 1 mL of sterile Phosphate-Buffered Saline (PBS) and drawing out approximately 1.5 mL of the faeces-PBS mixture. A negative control was performed for each sample. All samples (i.e., blood, cloacal solution) were frozen at -20°C for further analyses.

2.3. Microbiota analysis

a) PCR amplification and sequencing

Although samples were conserved in PBS, they all underwent an extra rinsing with sterile x1 PBS after thawing and before extraction to reduce the impact of urea as a potential PCR inhibitor. Bacterial DNA was extracted following the method of Qiagen DNeasy® PowerSoil Pro Kit (Qiagen, Venlo, Netherlands). Bacterial DNA was amplified by PCR targeting the V3-V4 hypervariable regions of the 16s rRNA, prepared in a sterile room dedicated solely to metabarcoding procedures. The 20 µL PCR solution contained 2X Master Mix AmpliTaq Gold (Fisher), 0.008X BSA, 0.25 µM forward primer (GTGYCAGCMGCCGCGGTAA) and 0.25 µM reverse primer (GGACTACNVGGGTWTCTAAT) and 2 µL DNA extract. The amplification profile used the following conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min 35 s and a final elongation step at 72°C for 10 min. Three PCR replicates identified by three different libraries were carried out to detect possible faulty PCRs (Udvardi et al. 2008). To monitor potential contaminants at each step of the lab work, extraction controls and negative PCR controls were included in all amplifications. Positive PCR controls were included to monitor the amplification performance. To obtain a unique tag combination for each PCR product, the primers were tagged using a system of 36 octamers with at least five differences between them. Sequencing controls (non-existent tag combinations) were included to detect potential tag-jumps. The library construction (PCR-free Bioscientific library preparation kit) and the sequencing (Illumina MiSeq 250 bp paired-end v3 chemistry) were performed at the Genopole of Toulouse (France).

b) Bioinformatics and data curation

Illumina sequencing data were processed and filtered using the "OBITools" package (Boyer et al. 2016). Forward and reverse reads were aligned with the *illuminapairedend* command. Alignments with a score below or equal to 40 were filtered out (Taberlet et al. 2018). Sequences were then assigned to samples using the *ngsfilter* command, allowing 0 and 2 errors on the tags and primers, respectively. Identical sequences were merged with the *obiuniq* command. Next, low-quality sequences were filtered out, i.e. sequences with less than one read in the whole dataset, sequences containing ambiguous bases or that were shorter than expected (<80 bp). Then, sequences were grouped into MOTUs (Molecular Taxonomic Operational Units) with *Sumaclust* using a similarity threshold of 97%. The taxonomic assignment for each MOTU was carried out with the SilvaNGS pipeline v.1.9.10 and the SILVA SSU database v138.1. Data curation was carried out using the R package "MetabaR" (Zinger et al. 2021). More specifically, PCR/sequencing errors, contaminant sequences, tag-jumps and dysfunctional PCRs were minimised. MOTUs with maximum abundance in the negative controls were considered contaminants, and those with a similarity to the reference sequence below 80 % were considered degraded sequences or chimaeras and were discarded. MOTUs that did not belong to the targeted clade (i.e., Bacteria) were also excluded. The noise produced by tag-jumps was reduced by removing a MOTU in a given PCR product when its relative abundance represented <0.03 % of the total MOTU abundance in the entire dataset. Finally, PCR replicates with a sequencing depth below a given threshold, and those that did not show good reproducibility were removed. The final MOTU table was produced by summing the reads of the PCR replicates originating from the same sample. To standardise differences in sequencing depth among samples (range: 4387 to 31100 reads per sample with an average of 17684.8 reads per sample), we rarefied all samples to a 4300 reads (Fig. S1) for the alpha diversity, beta diversity and the taxonomic composition analyses.

c) Metrics studied

Several alpha and beta diversity metrics were chosen to characterise the gut microbial communities. Alpha diversity was estimated using i) the number of different MOTUs present in each sample as a measure of specific richness and ii) the Shannon diversity index (H'), which considers the relative abundance of each MOTU within samples (Shannon 1948). Beta diversity (Whittaker 1960) was estimated using i) the Bray-Curtis index (based on a relative abundance matrix, Bray and Curtis 1957) and ii) the Jaccard dissimilarity index (based on presence/absence matrix, Jaccard 1912). Both indices vary between 0 and 1, with a high beta diversity index indicating a low level of similarity, while a low beta diversity index shows a high level of similarity. The matrices of the two indices were noticeably distinct (Fig. S2). Thus, we chose to use both indices to measure beta diversity.

2.4. Pesticides analysis

a) Laboratory analyses

A total of 116 molecules were assayed from blood samples, including herbicides, fungicides, insecticides, a synergist and a safener. These are active molecules (i.e., hereafter referred to as "pesticides") among the most used in France. Pesticide extraction was performed by concentrating samples and then subjecting them to multiresidue analysis using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and automated thermal desorption gas chromatography coupled to tandem mass spectrometry (ATD-GC-MS/MS) (see Rodrigues et al. 2023 for more details on methodology). The limits of detection (LOD) and quantification (LOQ) values of detected pesticides are provided in Supplementary material (Table S1).

b) Metrics studied

In addition to the concentration of each pesticide, the contamination level is commonly assessed in ecotoxicology studies by the number of pesticides detected and the total sum of pesticide concentrations (Tartu et al. 2014; Fritsch et al. 2022). Furthermore, as for the microbiota, we calculated a Shannon index per individual by transforming the concentrations to the nearest integer to represent the abundances of each pesticide, then, we studied beta diversity to assess the change in the diversity of pesticides from one profile to another. Thereafter, "pesticide load" will be used to refer generally to the level of pesticide contamination of individuals but will not target a metric unless it is specified.

2.5. Statistical analysis

Alpha (MOTU richness and Shannon index) and beta (Bray-Curtis and Jaccard indices) diversity indices were calculated with the R package "phyloseq" (McMurdie and Holmes 2013). The beta diversity analyses were performed with R using the "vegan" package (Oksanen et al. 2022). All the statistical procedures were performed using R version 4.3.2. (R Core Team 2021).

To evaluate the possible links between body condition and the different metrics of microbiota or pesticide load, we performed bootstrap-based Spearman's rank correlation tests to obtain 95% confidence intervals (95% CI). We also used this test to determine which pesticide load metrics to use for studying the link between microbiota and pesticides, i.e., the number of pesticides or the sum of the concentrations. Then, to study the change in microbiota alpha diversity (MOTU richness, Shannon index) according to the contamination status, we used generalised linear mixed effects models (GLMMs), including the sex, the body condition and the pesticide load in interaction as fixed effects and nest as random effect. MOTU richness followed a Poisson distribution, and the Shannon index was log-transformed to fit a Gamma distribution. The distributions were adjusted based on the *descdist* function from the "fitdistrplus" package (Delignette-Muller et al. 2023).

For microbiota beta diversity, we analysed the variance partitioning due to the interaction between sex, body condition, and pesticide load on the distribution of MOTUs based on dissimilarity matrices using PERMANOVA (ADONIS function; 1000 permutations). We also used Mantel tests to investigate correlations between the microbiota dissimilarity matrix (Bray Curtis & Jaccard) and pesticide concentration dissimilarity matrix (Euclidean distance). Then, an EnvFit analysis (1000 permutations) was performed to determine whether each pesticide affects microbial composition distribution (Holm-Bonferroni adjusted p-value < 0.05).

To study microbiota taxonomic composition and differential taxonomic abundances according to pesticide load, we used the ANCOM-BC (analysis of the compositions of microbiomes with bias correction; (Lin and Peddada 2020) function. We tested differential abundances of bacterial phyla and genera according to sex, body condition and pesticide load. All other parameters of the function were left on the default settings. Taxa with a Log Fold Change (LFC) value beyond >|1.5| and an adjusted p-value (Holm-Bonferroni) below 0.05 were considered differentially abundant.

The pesticide molecules detected in nestling blood had a very different range of concentrations (see Table S2 and Fig. S3). Moreover, some individuals had a very high sum of concentrations (see below in Table 1). So, we chose to centre and scale the concentrations by molecule that refers to relative concentrations before summing them to obtain an index of pesticide load. This index was then used as the pesticide load variable in statistical analyses (i.e., GLMM, PERMANOVA, ANCOM-BC), hereafter referred to as the "sum of standardised concentrations" in the results section. The range of this variable varied between −13.12 and 20.73, corresponding to a variation from a low pesticide load to a high pesticide load respectively. Moreover, this index was strongly correlated with the sum of raw concentrations, the number of pesticides and the Shannon index on pesticide diversity (see Fig. S4), reflecting the level of contamination of individuals.

3. RESULTS

3.1. Microbiota diversity and composition in nestling cloaca

From the 22 nestlings, we identified 139 distinct MOTUs, with on average (mean ± se) 26 ± 2 MOTUs per individual cloacal sample (26 ± 3 MOTUs per male and 26 ± 3 MOTUs per female). For the Shannon index, we had an average index of 1.93 ± 0.05 per individual cloacal sample (1.92 ± 0.09 per male and 1.94 ± 0.06 per female). Cloacal microbiota MOTU richness and Shannon index were not different among the 13 nests (Kruskall-Wallis tests: MOTU richness, $Khi^2 = 14.16$, df = 12, *p-value* = 0.29 and Shannon index, $Khi^2 = 8.72$, df = 12, *p-value* = 0.73). The relationship between individuals' body condition and microbiota metrics showed inverse trends depending on their sex (Fig. S4). Indeed, females with higher body condition harboured a higher MOTU richness and Shannon diversity of bacteria (Spearman's rank correlation test: $\rho^{\text{MOTU richness}} = 0.31$, 95% CI = [-0.42;0.91] , n = 11; $\rho^{\text{Shannon index}} = 0.61$, 95% CI = [-0.07;0.92], n = 11), unlike males for whom the relationship seemed to be inverse ($\rho^{\text{MOTU richness}} = -0.51$, 95% CI = [-0.85; 0.14] , n = 11; $\rho^{\text{Shannon index}} = -0.49$, 95% CI = [-0.88;0.15], n = 11).

Overall, three phyla (Proteobacteria, Actinobacteria & Firmicutes) were detected in all individuals, and to a lesser extent, the phylum Bacteroidota was present in more than 50% of the nestlings in five males and seven females (Fig. S5). The phylum Proteobacteria was the most prevalent and abundant in the cloacal microbiota of our individuals, representing more than 75% of the bacteria in all nestlings (Fig. S5). At the genus level, 90 genera were identified, with *Pseudomonas*, *Alcaligenes*, *Stenotrophomonas* and *Acinetobacter* being the most abundant and found in all nestlings (Fig. S6).

3.2. Pesticides detected in nestling blood

Overall, 36 of the 116 pesticide molecules were detected in the blood of the 22 nestlings, including 12 fungicides, 17 herbicides and seven insecticides (Fig. S3). Of the molecules found, 11 are supposed to be prohibited for use in France (Table S1). We identified on average (mean ± se) 11 ± 0.68 molecules per individual, with an average sum of the concentrations of 3494.38 ± 557.49 pg/mg and an average Shannon index of 1.91 ± 0.08 (see Table 1 for the description of contamination profiles by sex). Five pesticides were present in half or more of the nestlings: ethofumesate (n = 19), tebufenpyrad (n = 16), propiconazole (n = 14), lenacil (n = 11) and S-metolachlor (n = 11). Ethofumesate was present in all males and 8 of the 11 females. The number of pesticides, the sum of pesticide concentrations (raw concentration values) and the Shannon index on pesticide diversity in individuals were not different among the 13 nests (Kruskal-Wallis tests: Number of pesticides, Khi^2 = 11.20, df = 12, *p-value* = 0.51; Sum of concentrations, Khi^2 = 13.26, df = 12, *p-value* = 0.35; Shannon index, Khi^2 = 13.69, df = 12, *p-value* = 0.32). Body condition did not appear to be correlated with pesticide metrics (Fig. S4). Only one trend really seems to appear in males, with a positive relationship between their body condition and their pesticide diversity estimated by the Shannon index (ρ = 0.47, 95% CI = [-0.35;0.92], n = 11), although this is not significant (ρ = 0.47, *p-value* = 0.15). For the other relationships, Indeed, Spearman's rank correlation tests did not show any significant correlation between body condition and a) number of pesticides (Males: ρ = 0.17, 95% CI = [-0.55;0.81], n = 11; Females: ρ = 0.15, 95% CI = [-0.50;0.78], n = 11), b) sum of pesticide concentrations (Males: ρ = -0.28, 95% CI = [-0.80;0.46], n = 11; Females: ρ = -0.04, 95% CI = [-0.74;0.75], n = 11), c) Shannon index (Females: ρ = -0.29, 95% CI = [-0.85;0.44], n = 11).

Table 1. Pesticide load description in the 22 nestlings according to their sex. The sum of concentration is in pg/mg.

	Mean ± SE	[Min; Max]
Males (n = 11)		
Number of pesticides	10.64 ± 0.90	[5; 15]
Sum of concentration	2717.61 ± 641.32	[551.87; 6314.26]
Shannon index	1.86 ± 0.09	[1.33; 2.24]
Females (n = 11)		
Number of pesticides	11 ± 1.06	[6; 16]
Sum of concentration	4271.16 ± 879.43	[1510.30; 11772.86]
Shannon index	1.96 ± 0.12	[1.15; 2.41]

3.3. Relationship between the gut microbiota and pesticide contamination

Cloacal microbiota MOTU richness was significantly affected by the sum of the standardised concentrations in interaction with the sex and the body condition (GLMM: z value = -3.16, *p-value* = 0.002; Fig. 1, and see the summary of GLMM in Table S3, Supplementary Material). Indeed, the MOTU richness of the microbiota increased with the sum of standardised concentrations of pesticides only in females with a high body condition (group "High" in Fig. 1). Conversely, in males with a high body condition (group "High" on Fig. 1), microbiota richness decreased with increasing pesticide load. The result was the same with the Shannon diversity index, which was also influenced by the triple interaction between sex, body condition and the sum of standardised concentrations (GLMM: t value = -2.77, *p-value* = 0.006; see the summary in Table S3). The Shannon index representing the microbiota diversity increased with the sum of standardised concentrations of pesticides in females with a high body condition, while in males with a high body condition, it decreased.

Regarding the beta diversity, we did not find any effect of the interaction between sex, body condition and the sum of standardised concentrations of pesticide on microbial community structure (PERMANOVA: Bray-Curtis distances, F = 0.23, *p-value* = 0.94; Jaccard distances, F = 1.13, *p-value* = 0.27). The microbiota dissimilarity matrices (Bray-Curtis & Jaccard, respectively) were not correlated with the pesticide concentration dissimilarity matrix (Mantel: r = -0.14, *p-value* = 0.79 and r = 0.03, *p-value* = 0.41). The EnvFit analysis did not demonstrate any effect of specific pesticides on the microbial structure (Table S4).

Finally, regarding taxonomic composition, we separated our individuals into two groups of difference in pesticide load: low sum of standardised concentrations (Low pesticide load) and high sum of standardised concentrations (High pesticide load), ranked according to the median to have the same number of samples in each group. At the phylum scale, we only found a significant difference in the relative abundance of Actinobacteria between these two groups of individuals (Table S5), with more of this phylum in nestlings with a high pesticide load in their blood (nestlings with high pesticide load: median = 4 % of the microbiota; nestlings with high pesticide load: median = 2 % of the microbiota). However, the ANCOM analysis highlighted a significant effect of the sum of the standardised concentrations on one phylum, Bacteroidota (lfc = 0.09 ± 0.02, W = 4.06, *q-value* = 0.018). No effects of sex and body condition were found (Table S6). At the genus level, no effect of the variables tested (sex, body condition, sum of the standardised concentrations) was found with the ANCOM analysis on the ten main genera (Table S6). However, focusing on the ten most common genera (out of 90 detected), we found that individuals who had a lower pesticide load (on the left in Fig. 2), had a higher relative abundance of *Burkholderia* (in green, median = 4% of the microbiota; 0% for the "High pesticide load" group) and a lower relative abundance of *Leifsonia* (in brown, median = 2% of the microbiota; 4% for the "High pesticide load" group) in their microbiota (Mann-Whitney U tests: *Bulkholderia*, W = 88, *p-value* = 0.03; *Leifsonia*, W = 27, *p-value* = 0.03). We did not find any differences for other genera (Table S5).

4. DISCUSSION

To our knowledge, this study is the first to examine Montagu's harrier gut microbiota and provides evidence for the effect of exposure to a cocktail of pesticides on the gut microbiota of farmland bird according to their sex and body condition. Briefly, at a high phylogenetic level, the global composition of the gut microbiota is congruent with what is typically found in birds, featuring the four main phyla: Actinobacteria, Bacteroidota, Firmicutes and Proteobacteria. This last phylum was found predominantly in all nestlings; nevertheless, the genus *Burkholderia* detected more in individuals who had a lower pesticide load in their blood. Another phylum, Bacteroidota, was significantly more present in nestlings with a higher concentration of pesticides in their blood. Regarding the characteristics of nestlings, in high-body condition females, there was a positive correlation between pesticide load and gut microbiota diversity, while the relationship was the opposite in high-body condition males, with a lower microbial diversity when pesticide load was high. These results suggest that body condition exacerbates the effect that pesticides have on the microbiota richness and diversity in a sex-dependent manner.

4.1. Microbiota structure

The microbiota composition we found is common to those described in wild birds (Sun et al. 2022), with the main represented phyla being Proteobacteria, Actinobacteria, Firmicutes and Bacteroidota, in decreasing order of relative abundance. Firmicutes are often the phylum that dominates the avian cloaca microbiota (Waite and Taylor 2014; Grond et al. 2018; Wang et al. 2019), whereas in Montagu's harrier nestlings, Proteobacteria were the most present. However, this may depend on many factors and should not be generalised for all bird species. For instance, a study on another raptor, the Eurasian Kestrel (*Falco tinnunculus*), showed that the same four major phyla (Proteobacteria, Actinobacteria, Firmicutes and Bacteroidota) were found in their microbiota (investigated in faeces) but adults harboured higher abundances of Proteobacteria while nestlings exhibited higher abundances of Firmicutes and Actinobacteria (Guan et al. 2020). Another study showed that the cloaca microbiota of the insectivorous barn swallow (*Hirundo rustica*) contained Proteobacteria outnumbering Firmicutes by a 2:1 ratio (Kreisinger et al. 2015). Thus, the composition of the microbiota is dependent on factors related to the sampling site, the stage of host development and also the bird species studied, making general rules difficult to raise.

In the Montagu's harrier nestlings, Proteobacteria were the dominant phylum, and this result is reflected at the genera scale. Four genera were found in all nestlings, all belonging to the Proteobacteria phylum: *Acinetobacter*, *Alcaligenes*, *Pseudomonas*, and *Stenotrophomonas*, from which very few are currently known in the literature, especially in bird species. *Acinetobacter* or *Alcaligenes* species are usually commensal organisms, but they occasionally cause infections, as in Humans (Dahiru and Enabulele 2015; Wang and Xiao 2023). *Stenotrophomonas* spp. are often associated with soil and plants (Ryan et al. 2009), this genus containing opportunistic bacteria that are widespread within the environment (Kursa et al.

2022). Likewise, *Pseudomonas* includes bacteria widely distributed in the environment, particularly in soil and water, and thus commonly found in plants and animals. This genus includes bacteria with numerous functional and environmental implications (see Araos and D'Agata 2019), and some of them are associated with health issues, such as *Pseudomonas aeruginosa*, which is the most documented and important species affecting humans, responsible for serious life-threatening infections (Wretlind and Pavlovskis 1983; Nathwani et al. 2014). The increase of these pathogen bacteria in hosts could reflect host responses to environmental stressors, such as contamination by pesticides.

4.2. Pesticide contamination patterns

A wide range of pesticides was present in the studied nestlings, highlighting the agricultural context in which the nestlings are raised. The pesticide loads found in nestlings were variable, with some individuals being strongly contaminated (e.g., 15 pesticides in their blood and 11772.86 pg/mg) and others having lower contamination levels (e.g., five compounds and 551.87 pg/mg). Herbicides were most abundant in nestlings (17 of 36 compounds), with ethofumesate present in 19 of 22 individuals and dicamba present at the highest concentrations (Table S2, Fig. S3). All pesticide molecules found in individuals were considered non-toxic based on acute toxicity hazard classification, even for molecules that were found at high concentrations, such as dicamba, whose highest concentration (4272.08 ± 217.84 pg/mg) found in all individuals did not exceed 4% of the LD50 value (Table S1).

Montagu's harriers nest on the ground in cereal crops, which are dominant in the study area. Our results highlighted that naïve individuals such as nestlings might be contaminated by pesticide mixtures after only four weeks of life in crop plots. The quantities of substances applied on the study site and around the nests may vary depending on several parameters: quantity of pesticide to be applied per hectare, concentrations of active ingredients in the products, and type of crop. For example, a beet crop can lead to higher use of metamitron, while a corn crop will be treated with other herbicides such as dicamba. Overall, concentrations of different pesticides found in nestlings reflected the general pattern of pesticide use in the study area (Fuentes et al. 2024a, b) and would involve the persistence of substances for several weeks or months in crop plots, i.e., given that the treatments are mainly done before the reproduction of the harrier, starting in winter.

Moreover, the variability of pesticide load in nestlings could come from diverse exposure routes: ingestion of contaminated prey (e.g., voles), breathing and/or skin contact with contaminated soil, rain or crops (Moreau et al. 2022; Fuentes et al. 2024a, b). These diverse routes can be a source of variability in the contamination of nestlings, for example, with individuals that eat more and ingest more pesticides than others. Among the molecules detected in the blood of nestlings, metamitron and carbendazim are examples of molecules that had also been detected in micromammals in the study area, possible sources of food contamination in the Montagu's harrier (Fritsch et al. 2022). Pesticide ingestion can occur differently among individuals, depending on the type of prey ingested, for instance.

Finally, the global contamination of all nestlings is also worrying because we observed that several molecules found in the blood of chicks have been banned from use for several years, e.g., acetochlor, bifenthrin, quinoxyfen, terbutryn. This may suggest either fraudulent use, strong persistence in soils treated before the ban, or the detoxification process of females exposed during their wintering in Africa to pesticides, which are still authorised for use (Moreau et al. 2022). Our results, therefore, highlight the ubiquity of pesticides in agroecosystems, including some that have been banned for many years.

4.3. Potential role of pesticide contamination on gut microbial community shifts

Regarding the microbial composition of the nestlings, this seems to reflect their contamination by pesticides. Bacteroidota increased in the presence of a higher pesticide load in nestlings seems in agreement with the result of a study on Japanese quail, which had shown a positive relationship between Bacteroidota and pesticide load (Crisol-Martínez et al. 2016). Other experimental studies on Japanese quails have shown that the ingestion of trichlorfon, an organophosphate insecticide, induces an overall increase in Proteobacteria in microbiota, which can also be identified as an indicator of microbial dysbiosis in mammals (Round and Mazmanian 2009; Sobhani et al. 2011; Shin et al. 2015). Thus, the high presence of Proteobacteria in the nestling microbiota could be the consequence of the disturbance by all the pesticides and could become deleterious for the host. The high presence of this phylum could also reflect the plasticity/acclimatisation/adaptation of the host to the disturbance due to pesticides (Alberdi et al. 2016; Houwenhuyse et al. 2021). Among different phyla, Proteobacteria are known to harbour many pesticides degrading bacteria (Kumar et al. 2021), but Actinobacteria are also known to remove xenobiotics such as pesticides (Alvarez et al. 2017), which could also explain the higher abundance of this phylum in nestlings with a higher pesticide load in their blood. The high abundance of Actinobacteria is mainly explained by the members of the genus *Leifsonia*. Thus, the strong presence of Proteobacteria and Actinobacteria in nestlings could be an early adaptation of these individuals to an agricultural context where a wide range of pesticides is used.

Another genus caught our attention, *Burkholderia* (Proteobacteria), which was significantly more abundant in nestlings that had a lower concentration of pesticides in their blood. Commonly found in soil and associated with plants, *Burkholderia* includes certain bacterial strains such as *Burkholderia cenocepacia*, which are known to degrade methyl-parathion, an organophosphate pesticide, or lindane, and organochlorine pesticide, in soils (Mahenthiralingam et al. 2005; Fernández-López et al. 2017; Kumar 2018). In animals, *Burkholderia* spp. are gut bacteria common to a wide range of heteropteran insects, including major pests in agriculture (e.g., *Riptortus pedestris*) (Kikuchi et al. 2011). In these hosts, studies have highlighted the involvement of these bacteria in the degradation of pesticides, such as fenitrothion in stinkbugs, imparting protection against organophosphorous pesticides to these organisms as a symbiont (Singh 2009; Kikuchi et al. 2012). Insects are part of the diet of Montagu's harriers through direct (mainly orthopteran insects) or indirect consumption through voles, which are insectivorous (Trierweiler and Koks 2009; Werren 2012). Studying the diet of the Montagu's harrier nestlings through the composition of the rejection pellets to identify the different items and then the study of some prey (e.g., insects) could be the first step to verify if the prey that can be ingested by nestlings contains high abundances of *Burkholderia*.

4.4. Sex differences in gut microbiota responses to pesticide contamination

Male nestlings with a high body condition had lower microbial richness and diversity when they had a higher pesticide load, whereas for high body condition females, the relationship was reversed. This was not the case for nestling with a low body condition, regardless of their sex. Pesticides such as neonicotinoids or organophosphates have already been associated with a reduction in microbiota diversity (Crisol-Martínez et al. 2016; Han et al. 2023), which agrees with the observed pattern in males. In females, the opposite trend could be explained by physiological and particularly endocrine processes, which are different from those of males.

Given that Montagu's harrier is an altricial species, juveniles depend on food provided by their parents, and the larger sex, i.e., females (Arroyo et al. 2002), is usually more expensive to raise and have overall higher nutritional requirements, also showing higher growth and metabolic rates(Torres and Drummond 1999). Females with a higher body condition could thus modulate the toxic effect of pesticides on the microbiota and reduce these impacts by dilution or retention effect in adipose tissues (La Merrill et al. 2013). This would suggest a lesser impact of pesticides on the microbiota of females thanks to the presence of more adipose tissues, compared to males, which are generally smaller and have a less advantageous metabolism to reduce the effects of pesticides. Moreover, males would have not the advantage over females to acquire more food in the nest (Arroyo et al. 2002). This priority effect in resource acquisition could leave the males with fewer choices, with items rejected by females or with less food available, particularly when food is not abundant, and parents are taking smaller prey, such as insects instead of micromammals. This could thus suggest a difference in the sources of microbial colonisation between the sexes, i.e., females with a higher body condition ingesting more varied food items, which would increase their microbial diversity. While males would have fewer food choices and, therefore, less microbial diversity ingested, being more reflected in larger males.

4.5. Conclusion

This study highlighted for the first time a relationship between the exposure to a cocktail of pesticides and the variation in the composition of the gut microbiota in a farmland raptor, although correlative and obtained on a few individuals (due to the conservational status of Montagu's harriers). Given the crucial role of the microbiota have on its host health and physiology, such as vitamin synthesis, host metabolism, nutrient absorption, immune function, and organ development (Kohl 2012; Qin et al. 2012), it is mandatory to further investigate how pesticide cocktails act on this major physiological function. Our results thus open new perspectives to understand the contribution of pesticides in bird decline and, to a further extent, to the health of non-target organisms.

5. REFERENCES

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Ethical consideration

This work has received approval for research ethics from the "Centre de Recherches sur la Biologie des Populations d'Oiseaux" (CRBPO; Museum National d'Histoire Naturelle; licence #1308) and a proof of approval is available upon request. Bird captures, and experimental procedures conformed to the French guidelines for the ethical use of animals in research (APAFIS #36725-2022041910139000 v6).

CRediT authorship contribution statement

Léa Bariod: Writing – original draft, Formal analysis, Conceptualization**. Elva Fuentes:** Writing – review & editing. **Maurice Millet:** Writing – review & editing, Formal analysis. **Samuel Jacquiod**: Writing – review & editing. **Joël White:** Writing – review & editing, Formal analysis, Conceptualization. **Jérôme Moreau**: Writing – review & editing, Supervision, Conceptualization. **Karine Monceau**: Writing – review & editing, Supervision, Funding acquisition, Project administration, Conceptualization.

Data Availability

Data will be made available on request.

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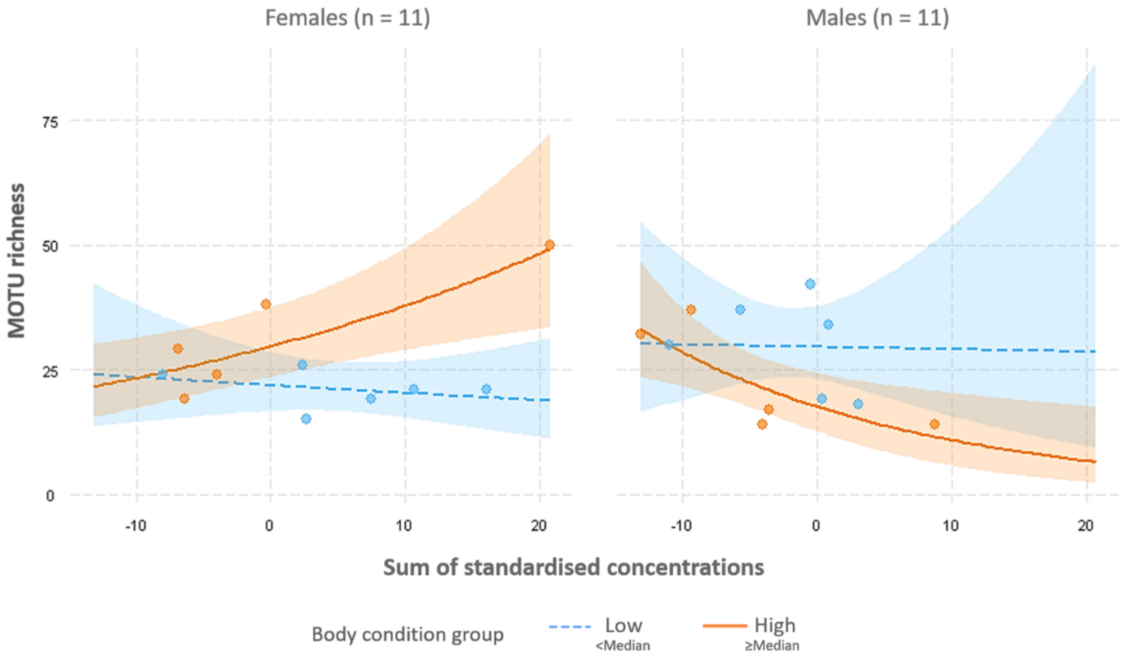

Figure 1. Relation between MOTU richness and the sum of standardised concentrations according to the body condition and by sex. For greater visibility of the results, we divided our individuals into two classes of body conditions established by sex around their median condition: Group "Low" in blue, below the median corresponding to a low body condition (males: [245.26 g; 288.12 g[, females: [283.25 g; 313.07 g]); Group "High" in orange, above the median corresponding to a high body condition (males: [288.12 g; 358.03 g[, females: [313.07 g; 372.51 g]).

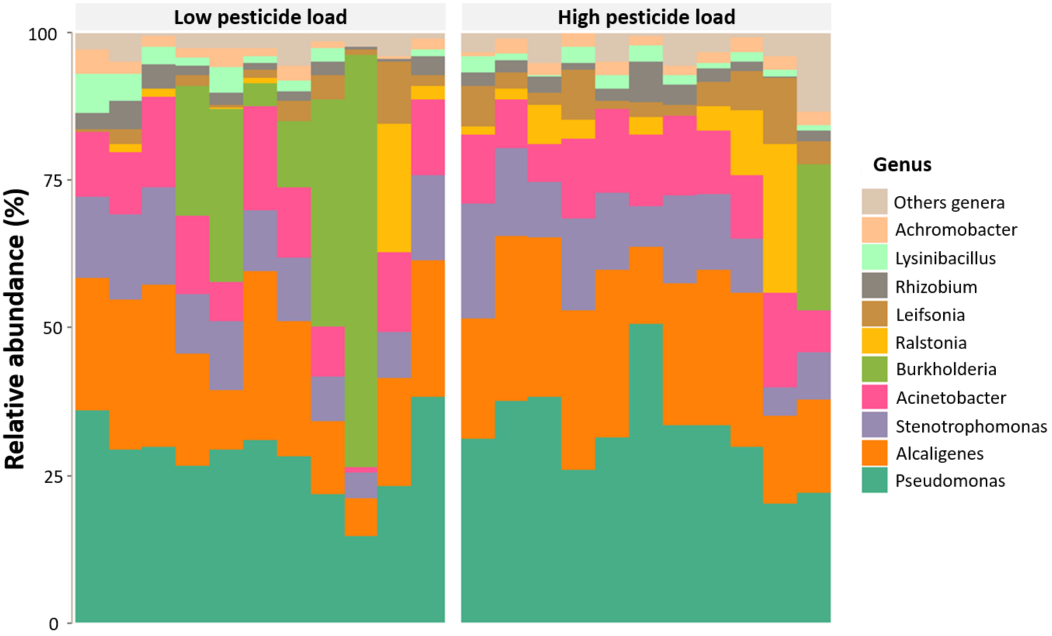

Figure 2. Relative abundance of the ten most common genera found in the microbiota of Montagu's harrier nestlings according to their pesticide load. "Low pesticide load" corresponds to the load below the sum of the standardised concentrations median, and "High pesticide load" corresponds to the load above the sum of the standardised concentrations median.

Contamination par les pesticides des poussins de busard cendré, *Circus pygargus* : patrons, facteurs d'influence et effets sublétaux

Résumé : Le déclin actuel des oiseaux spécialistes des terres agricoles dans le monde entier a principalement été attribué à l'utilisation d'intrants synthétiques, en raison de leurs effets directs et indirects sur les organismes non-cibles. Cependant, d'importants manques de connaissance persistent, notamment sur les effets cocktail des pesticides sur les oiseaux sauvages des terres agricoles *in natura*. Mon travail de recherche vise à comprendre comment les pesticides de synthèse peuvent affecter les oiseaux des terres agricoles en tirant parti du suivi à long terme des poussins de busard cendré *Circus pygargus* dans la Zone Atelier Plaine & Val de Sèvre (ZAPVS) et d'une nouvelle méthode d'analyse multirésidus, permettant de rechercher plus d'une centaine de substances dans le sang des oiseaux. L'approche utilisée ici m'a permis de mettre en évidence que (i) la proportion d'agriculture biologique dans les agroécosystèmes peut avoir à la fois des avantages et des inconvénients sur les traits d'histoire de vie des oiseaux des terres agricoles ; (ii) les mélanges de pesticides sont omniprésents dans le sang des oiseaux sauvages et ne dépendent ni des caractéristiques intrinsèques (rang d'éclosion, sexe ou condition corporelle) ni de la date d'échantillonnage, mais les niveaux de contamination sont réduits en fonction de l'âge ; et (iii) il n'y a pas d'effet sublétal majeur des mélanges de pesticides sur la croissance des poussins sauvages, mais plutôt des effets dépendant de l'âge et du sexe. Par ailleurs, mes travaux ont montré que l'agriculture biologique, en réduisant le nombre de pesticides dans le sang des oiseaux, réduit par conséquent le risque d'effets cocktail sur leur santé. Ces nouvelles connaissances soulèvent des questions concernant la pression-exposition des pesticides sur les oiseaux sauvages des terres agricoles dans les agroécosystèmes et souligne la nécessité de prendre en compte un large éventail de traits d'histoire de vie pour évaluer l'exposition-impact des pesticides sur les oiseaux sauvages. Dans l'ensemble, mon travail a apporté des éléments nouveaux et importants pour comprendre la complexité des facteurs influençant le devenir des pesticides chez les oiseaux des terres agricoles et les effets sur leur santé, un enjeu crucial pour la santé de l'Homme et de son environnement dans un contexte d'une seule santé (*One Health*).

Mots clés : Biosurveillance, effets cocktail, écotoxicologie, oiseaux des terres agricoles, agriculture biologique, mélanges de pesticides

Pesticide contamination in nestlings of Montagu's harrier, *Circus pygargus*: patterns, factors of influence and sublethal effects

Abstract: The actual decline of farmland birds worldwide has been attributed mainly to the use of synthetic inputs, due to their indirect and direct effects on non-target organisms. However, important knowledge gaps persist, notably on the cocktail effects of pesticides on wild farmland birds *in natura*. My research work aims to understand how synthetic pesticides may affect farmland birds by taking advantage of the long-term monitoring of Montagu's harrier *Circus pygargus* nestlings in the Zone Atelier Plaine & Val de Sèvre (ZAPVS) and of a new multiresidue analytical method, screening more than a hundred substances in birds' blood. The approach used here enabled me to evidence that (i) the proportion of organic farming in agroecosystems may have both benefits and drawbacks on the life-history traits of farmland birds; (ii) pesticide mixtures are ubiquitous in wild birds' blood and neither relies on intrinsic characteristics (hatching rank, sex or body condition) nor the sampling date, but that contamination levels are reduced in an age-dependent manner; and (iii) no major sublethal effect of pesticide mixtures on the growth of wild nestlings occurs, but rather age and sex-dependent effects. Moreover, my work evidenced that organic farming, by reducing the number of pesticides in birds' blood, reduces the associated risk of cocktail effects on their health. This new body of knowledge opens questions concerning the pressure-exposure of pesticides on wild farmland birds within agroecosystems and highlights the need to consider a wide range of life-history traits to assess the exposure-impact of pesticides on wild birds. Overall, my work provided novel and major insights into understanding the complexity of factors influencing pesticide fate in farmland birds, and the effects on their health, a crucial issue for human and environmental health, in a *One Health* context.

Keywords: Biomonitoring, cocktail effects, ecotoxicology, farmland birds, organic farming, pesticide mixtures

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