Pesticide mixture effects on physiological stress and morphology of growing wild nestlings

Elva Fuentes [a,*] , Jérôme Moreau [a], Maurice Millet [b] , Vincent Bretagnolle [a,c],
Karine Monceau [a]

[a] *UMR 7372, Centre d'Études Biologiques de Chizé, La Rochelle Université & CNRS, Villiers en Bois 79360, France*
[b] *Université de Strasbourg, CNRS-UMR 7515, ICPEES, Strasbourg cedex 2, 67087, France*
[c] *LTSER "Zone Atelier Plaine & Val de Sèvre", CNRS, Villiers-en-Bois 79360, France*

HIGHLIGHTS

- The effects of pesticide mixtures on wild birds' growth and stress are understudied.
- 35 Montagu's harrier chicks were monitored during the rearing period.
- Depressed baseline corticosterone was found in more contaminated nestlings.
- A sex and age-dependent effect of pesticides on chicks' growth was found.
- Trade-offs between detoxification, growth and corticosterone may occur *in natura*.

GRAPHICAL ABSTRACT
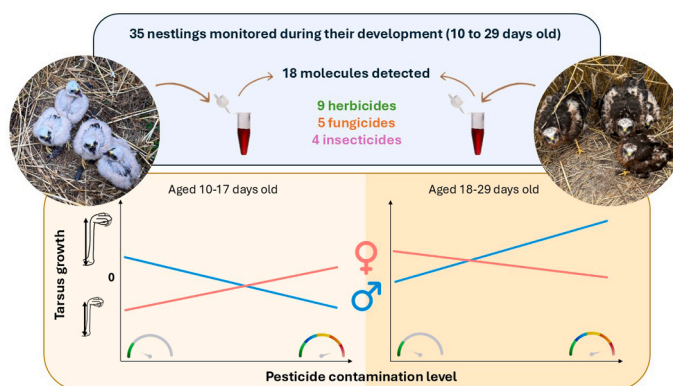
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ABSTRACT

Synthetic pesticides may pose a risk to non-target organisms, notably farmland birds, through their hazardous effects on life-history traits, but their impacts on growth and physiological stress have never been assessed *in natura*. We investigated these effects on skeletal growth, mass gain, body condition and physiological stress (basal and stress-induced corticosterone secretions) of 35 nestlings of *Circus pygargus*, a farmland apex predator, blood-sampled twice during their development. We found contamination levels ranging from 0 to 9 pesticides detected out of the 116 searched. We observed an age and sex-dependent pattern of pesticide mixture effects on nestlings' growth and baseline corticosterone. Males ($\leq$17 days old) having shorter tarsus when their pesticide load was high while this was found in older females ($\geq$18 days old). Higher pesticide loads were linked to poorer body condition of young nestlings irrespective of their sex. Similarly, depressed corticosterone secretion was found in more contaminated young nestlings, and in females irrespective of their age. These alterations observed in young nestlings are caught up later during their development. No relationship between contamination levels and stress-induced corticosterone was found. Including other life-history traits would provide more information

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* Corresponding author.
  E-mail address: elva.fts@gmail.com (E. Fuentes).

on other stress endpoints and potential energetic trade-offs between an active defence against pesticides and important functions of the organism.

1. Introduction

Synthetic pesticides and fertilizers used in conventional agriculture have been pointed out as the main factors driving farmland birds' population decline [81]. 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 [68, 70] for reviews), the toxicity of currently used pesticides, supposed to be of low-risk, is still under debate [17,80,92]. Yet, non-persistent pesticides (i.e., not considered as POPs) may have both indirect effects on birds through the reduction of resources [32] and direct effects through lethal or sublethal effects (i.e., affecting the organism in the long term; [70]). Besides, most studies considering non-persistent pesticide effects usually investigated the effects of a single class of pesticides under experimental conditions (but see mixtures of different classes on *Apis mellifera*, [1]; triazole fungicides mixtures on *Alectoris rufa*, [20]), neglecting the effects of complex mixtures of more than one pesticide class *in natura*. Although challenging, there is a need to investigate the underlying mechanisms linking pesticide contamination levels to farmland birds' decline under field conditions [70].

A developmental period is a critical period for organisms, being highly demanding in energy and implying many endocrine systems for its regulation [64,93]. 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 [64,87,94]. 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, [34]). A chronic increase in corticosterone levels may result in fat store depletion, muscle loss, growth and immune system suppression [9]. 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 [46]. Although it is acknowledged that this mechanism allows hierarchies among siblings to be maintained [58], high corticosterone levels in juveniles might result in long-term consequences later in adulthood, notably on personality [87]. Some environmental pollutants can disrupt an individual's homeostasis by acting as endocrine disruptors which may further result in altered growth [33,9]. Depending on the contaminant, exposure of nestlings may either increase or inhibit their stress responses [8,22,23]. For instance, organochlorine and organophosphate insecticides may interfere with the hypothalamic-pituitary-adrenocortical (HPA) axis by disrupting the production or clearance of corticosterone [9]. 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 [69]. Therefore, exposure to contaminants during nestling development might slow down their growth and even increase their mortality [90]. For instance, barn owls (*Tyto alba javanica*) nestlings fed with prey contaminated with rodenticides were smaller than nestlings fed with uncontaminated prey [71]. Besides, house sparrow chicks from parents exposed to tebuconazole had reduced growth and higher mortality rate than chicks from the unexposed group [12]. 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 [28]. However, at 24-days old they caught up in size, but their body mass index was lower, suggesting that parents' exposure to pesticides might have affected chicks' mass gain but not structural growth [28]. In red-legged partridges (*Alectoris rufa*), chicks from

parents fed with thiram-treated grains displayed slower growth rates than control chicks [54] while growth rates of chicks were unaffected when parents were fed with grains coated with a mixture (imidacloprid, thiram and difenoconazole [55]) or with imidacloprid alone [56]. Therefore, the effects of indirect nestling exposure (i.e., through parental exposure) on chicks' growth may vary depending on the pollutants concerned. Still, a shared shortcoming in all these studies is that pesticide titration was not realized during chicks' growth, thus limiting the direct association between their contamination levels and 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 [43]. Moreover, Bonelli's eagle (*Aquila fasciata*) chicks with high levels of DDT in their blood had reduced body condition [73], and bromadiolone detection in the blood of fledglings of common kestrel (*Falco tinunculus*) was associated with lower body mass [61]. However, there is a global 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 [63].

Farmland birds currently breeding in agricultural fields of Europe and other intensively managed areas 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, [16] or red-legged partridges, [72]) and maternal transfer of compounds from females to their eggs (e.g., [11]). In Montagu's harrier, nestlings were recently found to be contaminated with pesticide mixtures of different classes and the basal contamination levels of hatchlings decreased as nestlings got older, probably due to the development of their detoxification capacities [25,26]. Nestlings might be 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, although the contribution of each pathway is not quantified to date [26]. Moreover, Montagu's harrier displays hatching asynchrony (1–2 days between hatching of subsequent eggs in a brood) and sexual dimorphism, with females being heavier than males (~19 % visible during the second part of the rearing period; [67]), leading to hierarchies among nestlings. First-hatched chicks and females are generally advantaged in sibling competition for food within a brood due to their larger size [3], although the sex of chicks does not seem to influence their corticosterone production [78]. 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 four 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 [35].

2. Materials and methods

2.1. Ethical statement

The handling of Montagu's harrier chicks received approval 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). A certificate of approval is available upon request.

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 km². The area is predominantly covered by intensively managed cereal crops (~41 % of the cultivated area between 2009 and 2016; [15]). 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).

2.2.2. Model species

The Montagu's harrier has been monitored in the ZAPVS since 1994 [15]. The individuals of the population studied in this area overwinter in western Africa and nest on the ground in cereal crop plots, laying up to five eggs (exceptionally eight; [7,29]). The incubation lasts 29 days and the rearing period 30–35 days [4]. Montagu's harrier breeding success depends mainly on the availability of its main prey, the common vole (*Microtus arvalis*), although they also prey upon orthopterans and passerine birds [86]. The breeding success in this site is ~2.05 fledglings per breeding attempt [6], with brood sizes varying between one and five nestlings (see [27] for an overview of several years). Males ensure food

provisioning of incubating females and chicks, hunting within home ranges of ~14 km² in the study area [85]. Females contribute to the food provisioning of chicks by the end of the rearing period, hunting close to the nest [30].

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; [76]; 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 $p = 1.00$).

2.3.1. Experimental design

During the hatching visit, chicks were head-marked using water-based coloured pens, allowing individual identification (Fig. 2a). Morphometric measures of chicks were taken during all nest visits (from their first days post-hatching until fledging at ~30 days-old) 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 2 min 20 s ± 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 (on average at 21 min 23 s ± 7 min 39 s) before
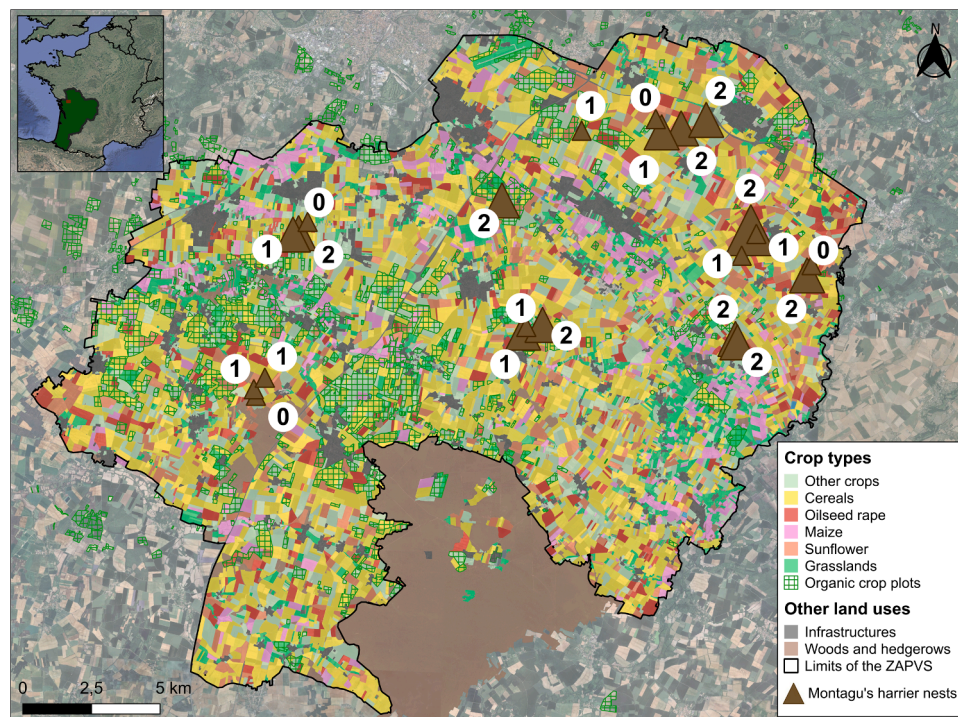

Fig. 1. Localisation of the 22 nests of Montagu's harrier (*Circus pygargus*) monitored in 2018 in the Zone Atelier Plaine & Val de Sèvre (ZAPVS). The size of the triangles reflects the number of siblings from the brood sampled for pesticide analyses (up to 3 chicks). Number in white circles indicates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

Fig. 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. The first developmental period corresponds to pictures (a) and (b), and the second to pictures (c) and (d). Photo credit: E. Fuentes.
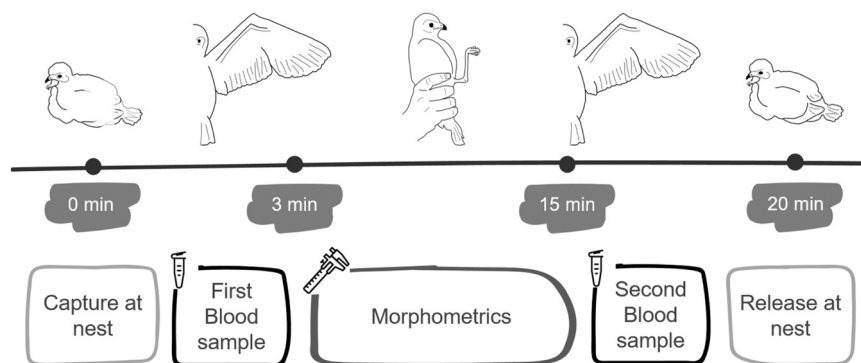

Fig. 3. Procedure followed during the second and fourth visits at Montagu's harrier (*Circus pygargus*) nests, when chicks were ~16 and ~26 days-old respectively.

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; [52]). The age of nestlings was estimated *a posteriori*, using a morphometric estimation of age (function using wing length measure [2]; calculator available at https://busards.com/index.php/Outils/biometry).

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 [66] to mean 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; [66]) of mean 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). Therefore, positive residuals stood for chicks having larger morphometrics than expected from the theoretical growth curve, while negative residuals indicated that chicks had smaller than expected morphometrics. 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 mass index (SMI) following Peig & Green [75], calculated for males and females separately. This index is supposed to reflect fat storage relative to the size of the individual and is less susceptible to variations due to recent food ingestion than the raw body mass. The body condition of chicks tends to decrease with age because of the reduction of body fat to attain the optimum mass for flight [62,95], thus we extracted the residuals of the linear regression of SMI on age (one for each sex; see Figure S3 in supplementary materials). These residuals (positive for an excess of fat stores and negative for a deficit) 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 maximal corticosterone (see Section 2.3.2.2) 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 (chicks of 16 ± 3 days-old) and fourth (chicks of 26 ± 2 days-old) visits. Among these chicks, some had no blood sample available for corticosterone titration, either for an entire visit (one nestling), the first blood sample of a visit (4 nestlings) or the second blood sample of a visit (3 nestlings). Thus, 27 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. [57], from 30 μL of plasma, corticosterone was extracted using ethyl ether and titrated by radioimmunoassay. The minimal detectable concentration of corticosterone was 0.28 ng.mL^{-1}. Three concentrations of corticosterone (ng.mL^{-1}) per chick per visit were obtained: the baseline corticosterone (BASECORT, blood collected when arriving at nest), the maximal corticosterone (MAXCORT, blood collected after handling) and the stress-induced response in corticosterone (REPCORT which is the MAXCORT minus the BASECORT). Mean BASECORT among the 65 samples was 5.82 ng.mL^{-1} $\pm$ standard deviation (SD) 4.15 ng.mL^{-1} for MAXCORT among the 66 samples mean $\pm$ sd was 28.86 ± 11.64 ng.mL^{-1}, and for REPCORT among the 62 complete sets of MAXCORT-BASECORT, 23.32 ± 11.20 ng.mL^{-1}. The BASECORT and REPCORT were not correlated to each other (Pearson's

correlation test, $r = 0.006$, 95 % CI = [-0.26; 0.29]). Although the MAXCORT was correlated to both BASECORT ($r = 0.35$, 95 % CI = [0.09; 0.58]) and REPCORT ($r = 0.94$, 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 [59,93], 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 (positive residuals) or deficit (negative residuals) 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. [82], 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 multiple reactions monitoring 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. [82] 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. [82].

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 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 concentrations of each substance were scaled for each sex separately prior to summation (for both total and most prevalent substances), to take into account the different concentration ranges and make the sum more equilibrated between pesticides. To define the most prevalent molecules, we chose the 25 % of molecules with the highest prevalence (the first quartile with higher prevalence among the 18 molecules). In other words, the most prevalent molecules were defined as those detected in at least 35 % of the samples (see Table 1). This threshold was set based on the distribution of our data as there is no study, to our knowledge, using a metric of "most prevalent pesticides" to assess mixture effects on health parameters (either human or wildlife). The number and sum of concentrations of the most prevalent molecules included picloram, chlorpyrifos-methyl, fenpropidin, S-metolachlor, and metamitron. These molecules, which accounted for > 50 % of the total detection frequency, were the most prevalent when considering both total prevalence and males and females prevalence independently. This approach allows to target the most frequently detected pesticides in the population studied, while limiting the analysis

Table 1

Pesticide molecules detected in the blood of 35 Montagu's harrier (*Circus pygargus*) chicks in 2018 (ordered by prevalence), including their target (F = fungicide, H = herbicide and I = insecticide), their chemical family, their mode of action (ModA), and the date of the ban in France, if any. Bold italics indicate the most prevalent molecules included in the metrics of contamination levels (number and sum of concentrations).

Molecule	Type	Family	ModA	Ban	Prevalence (in %)		
					Total	1st sample	2nd sample
Chlorpyrifos-methyl	*I*	*Organophosphate*	*AChE[c] inhibitors*	*2020*	*61.4*	*77.1*	*45.7*
Fenpropidin	*F*	*Piperidine*	*Inhibition of sterol biosynthesis*		*48.6*	*65.7*	*31.4*
Picloram	*H*	*6-Chloropicolinates*	*Auxin mimics*		*45.7*	*34.3*	*57.1*
Metamitron	*H*	*Triazinone*	*Photosynthesis inhibitor*		*40.0*	*45.7*	*34.3*
Metolachlor-S	*H*	*Chloroacetamide*	*Inhibition of VLCFA[d] synthesis*	*2024*	*35.7*	*34.3*	*37.1*
Cypermethrin	I	Pyrethroid	Sodium channel modulators		32.9	65.7	-
Ethofumesate	H	Benzofuran	Inhibition of VLCFA[d] synthesis		27.1	42.9	11.4
Carbaryl	I	Carbamate	AChE[c] inhibitors	2007	25.7	51.4	-
Dicamba	H	Benzoates	Auxin mimics		24.3	48.6	-
Fenpropimorph	F	Morpholine	Inhibition of sterol biosynthesis	2019	20.0	-	40.0
Penconazole	F	Triazole	Ergosterol-biosynthesis inhibitor		17.1	-	34.3
Prosulfocarb	H	Thiocarbamate	Inhibition of VLCFA[d] synthesis		17.1	34.3	-
Acetamiprid	I	Neonicotinoid	nAChR[a] competitive modulators	2018	15.7	-	31.4
Bupirimate	F	Pyrimidine	Nucleic acid metabolism		15.7	31.4	-
Propazine	H	Triazine	Photosynthesis inhibitor	2002	14.3	-	28.6
Aclonifen	H	Diphenylether	Inhibition of SDPS[b]		8.6	17.1	-
Dichlobenil	H	Nitrile	Inhibition of cellulose synthesis	2008	5.7	11.4	-
Thiamethoxam	I	Neonicotinoid	nAChR[a] competitive modulators	2018	1.4	-	2.9

[a] nAChR: Nicotinic Acetylcholine receptor
[b] Solanesyl DiPhosphate Synthase
[c] Acetylcholinesterase
[d] Very Long-Chain Fatty Acid

to a small number of molecules to avoid dilution of the signal by low-presence substances. Although the four metrics moderately to strongly correlate to each other (Pearson's correlation coefficient between 0.51 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). Although these metrics do not take into account the relative toxicity of each substance, the toxicity endpoints used in other metrics such as the toxic unit are questionable [70]. Besides, to date, there is no metric allowing to account for the complexity of pesticide mixtures (synergist or agonist effects; [39]), therefore we chose to rely on the concentration addition principle, assuming that the higher the contamination, the larger the risks of adverse cocktail effects.

The residuals of growth parameters (mean 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 random variable "nest" also enables to take into account differences in diet between nestlings of different nests as adults may display different hunting patterns (some would bring more voles, others more alternative prey as passerine birds or orthopteran insects).

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 10–17 days and the second corresponding to ages of 18–29 days. These classes were defined according the sigmoidal growth pattern of semi-altricial nestlings that display a rapid structural growth during the first half of the rearing period (Fig. 2a, b) and then a level off by the second period that coincides with the period of rapid growth in feathers (Fig. 2c, d) ([44]; but see [5,18,47] 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 figures 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 [58].

All models were implemented using the package 'lme4' [10] and models residuals were checked using the package 'DHARMa' [38] in R v.4.2.2 software [77]. 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' [21] 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' [53]. Futhermore, we performed a power analysis to determine the model robustness and generalizability of the results using 'simr' package [36] and provided the results in supplementary materials (Figure S6).

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 scaled concentrations ($\sum tot$) or among the most prevalent

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
Scaled $\sum tot$	0.00 ± 4.99	−7.47	13.29
Scaled $\sum preval$	0.00 ± 2.02	−2.85	4.83

($\sum$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. [25].

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

The total sum of scaled compound concentrations in blood ($\sum$tot) in interaction with chicks' sex and age significantly explained chicks' tarsus length (LMM, χ^2=4.90, d.f.=1, P < 0.05, Table S4). This triple interaction was also found for the sum of scaled concentrations of the most prevalent pesticides ($\sum$preval; LMM, χ^2=4.25, d.f.=1, P < 0.05, Table S5). During the structural growth period (10–17 days), males with higher $\sum$tot had delayed tarsus growth (i.e., negative residuals) compared to low-contaminated males (Fig. 4). Females had a reversed relationship, although they mainly kept a deficit in tarsus length compared to the expected tarsus growth curve whatever their pesticide concentrations (Fig. 4). These patterns were reversed in the second part 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). Similar patterns were found for $\sum$preval: reversed relationships between males and
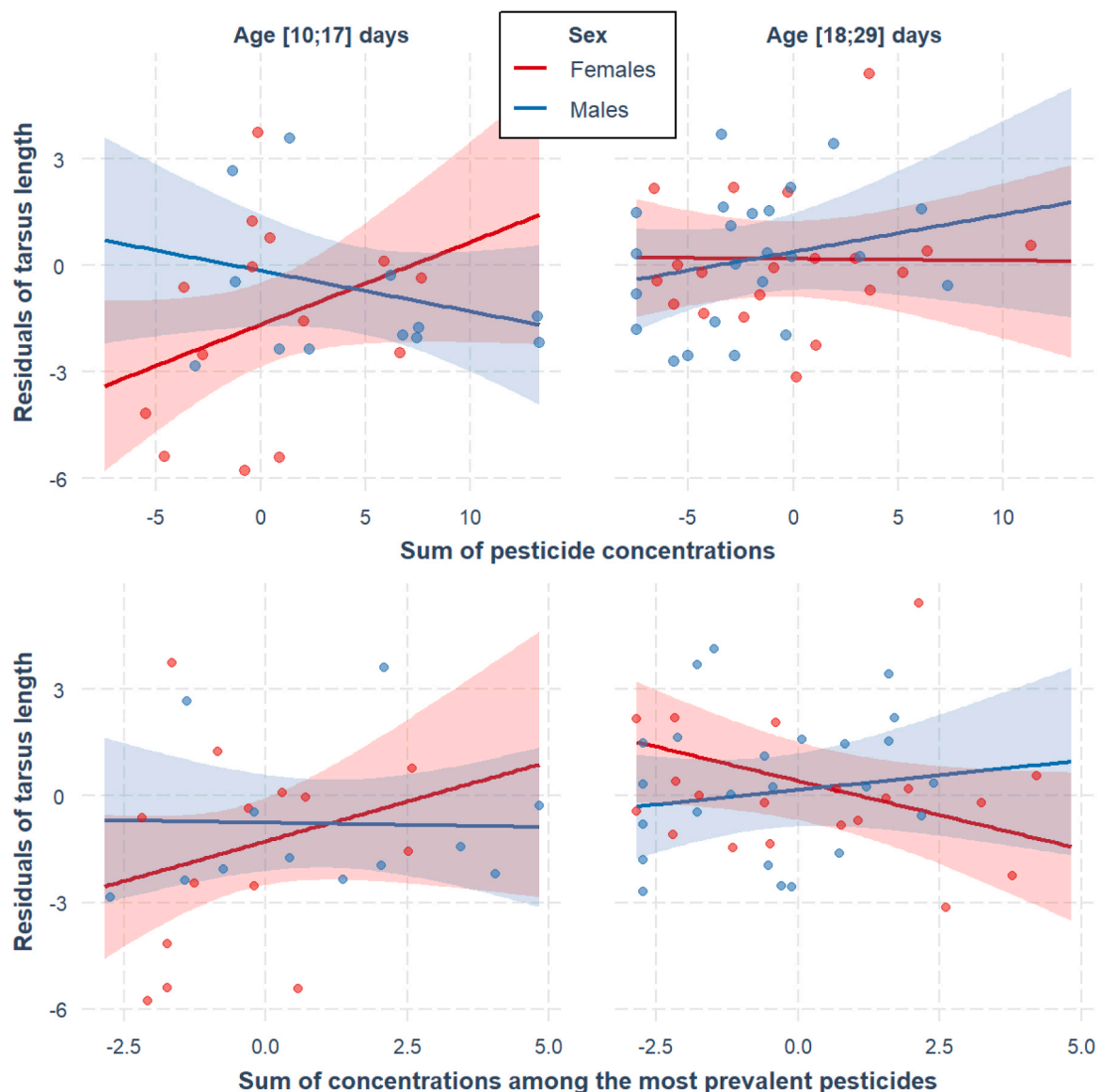

Fig. 4. Effect of the triple interaction of pesticide contamination and nestlings' sex and age on their residual tarsus length. Sum of pesticide concentrations (top) and of the most prevalent pesticides (bottom) were obtained by scaling substances' concentrations prior to summation. Chicks sampled during 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.

females and among the two rearing periods (Fig. 4).

3.2.2. Mass gain

None of the pesticide contamination metrics significantly explained the mass residuals of Montagu's harrier nestlings, only age explained their 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 explained chicks' body condition (Table S2, S3, S4 and S5). However, the sum of concentrations of the most prevalent compounds ($\sum$preval) in interaction with nestlings' age explained the residuals of body condition (measured through the SMI) (LMM, χ^2=4.35, d.f.=1, $P < 0.05$, Table S5). Regarding the most prevalent pesticides, whatever their sex, the more contaminated were the nestlings, the larger their deficit in body condition (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).

3.2.4. Corticosterone

The scaled sum of concentrations of the most prevalent compounds ($\sum$preval) in interaction with sex explained chicks' baseline corticosterone (LMM, χ^2=4.10, d.f.=1, $P < 0.05$, Table S5). Moreover, this contamination metric in interaction with nestlings' age also explained chicks' baseline corticosterone (LMM, χ^2=5.49, d.f.=1, $P < 0.05$, Table S5). Whatever their age, the more contaminated the females, the lower their secretion in baseline corticosterone compared to the expected secretion, while males displayed a reversed relationship (Fig. 6). Whatever their sex, younger nestlings (~15 days old) had delayed corticosterone secretion when more contaminated while this trend was not found in older nestlings (~26 days old; Fig. 6). The other metrics of contamination (N_{tot}, N_{preval} and $\sum$tot) did not explain chicks' baseline corticosterone secretion (Table S2, S3, S4). There was no effect of the contamination levels, chicks' age and sex, brood size or hatching order on either 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 explained by their contamination with pesticide mixtures. The skeletal growth of chicks can be affected by the sum of pesticide concentrations in blood, notably males during the phase of rapid structural growth (up to 17 days old) showing shorter tarsus length when they have 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, with older females having shorter tarsus length when more contaminated. Whatever their sex, chicks with a high sum of concentrations (among the most prevalent pesticides) had lower body condition, although this effect was observed only for the first stage of their growth ($\leq$ 17 days old). More contaminated females (sum of concentrations among the most prevalent pesticides) had lower baseline corticosterone secretions, although this was reversed for males. Whatever their sex, young nestlings (~15 days old) had lower baseline corticosterone secretions when they had higher sum of pesticide concentrations (among the most prevalent). However, we did not detect any relationship between contamination levels and the mass gain or the stress-induced corticosterone secretions of nestlings.

Growth rates are mediated through the energy provided by food intake and hormones [64]. 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. In the present study, the effects of pesticide mixtures found on both Montagu's harrier chicks' growth and physiological stress are quite complex. Concerning chicks' physiological stress, we detected an altered secretion of baseline corticosterone for females and for younger nestlings with more contaminated nestlings having reduced corticosterone concentrations in blood. This result is consistent with findings on other pollutants such as polychlorinated biphenyls [60] or mercury [23,40]. However, few studies investigated corticosterone disruptions linked to the effects of non-persistent pesticides as the ones found here (but see [65] for an example on a reptile), and even fewer on birds [42]. Considering the three more contaminated nestlings (sum of concentrations among the most prevalent substances) with depressed baseline corticosterone (negative residuals), three
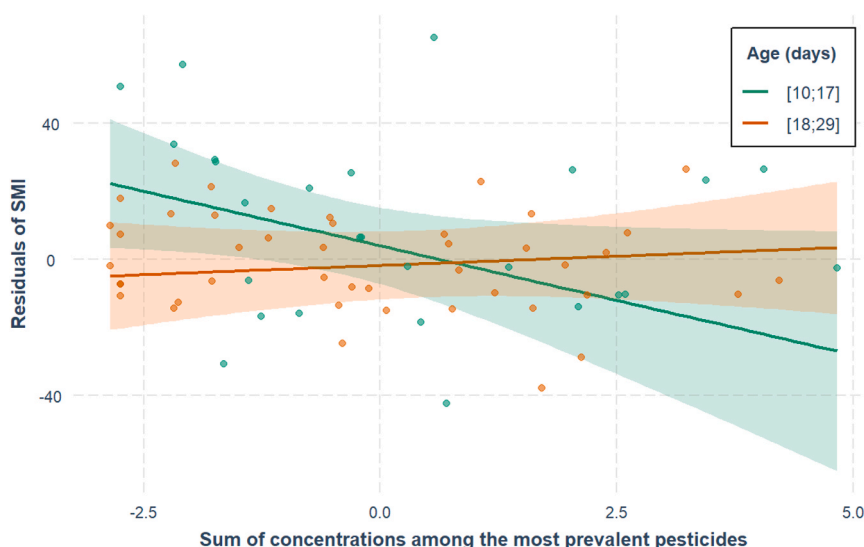

Fig. 5. Effect of the double interaction of pesticide contamination and nestlings' age on their residual body condition (measured through the Scale Mass Index, SMI). Sum of concentrations of the most prevalent pesticides (bottom) were obtained by scaling substances' concentrations prior to summation. 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.
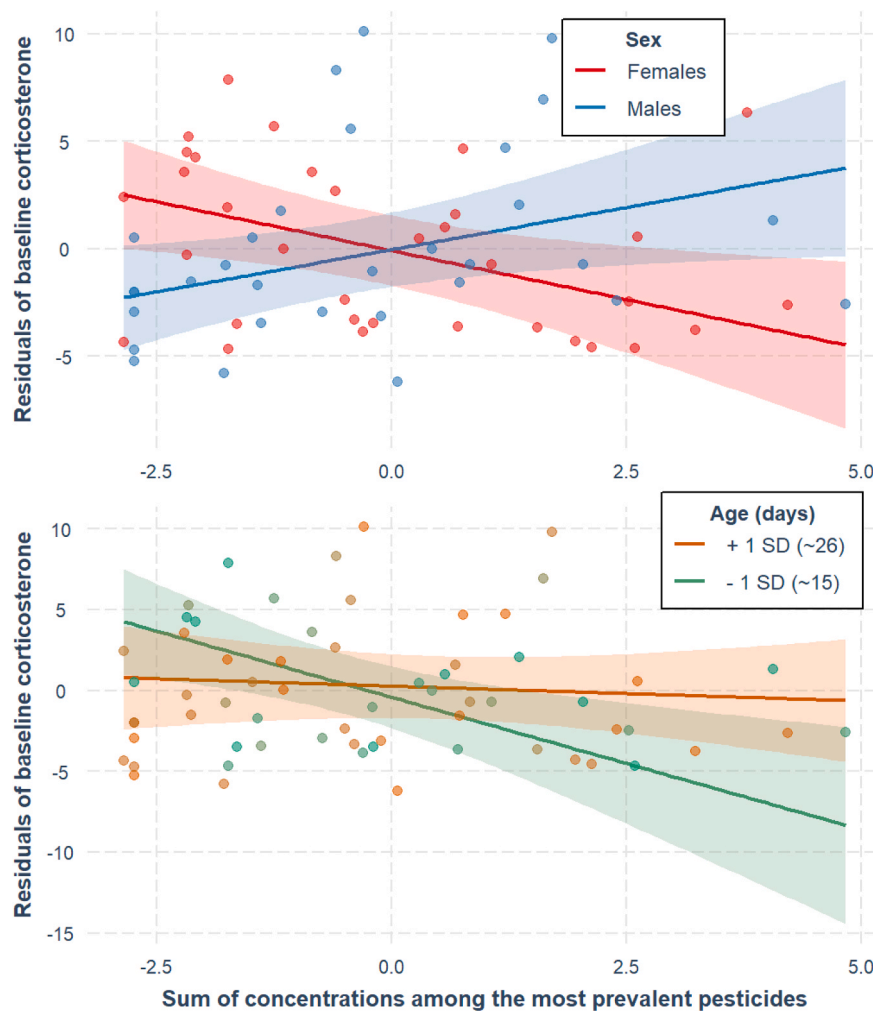
Fig. 6. Effect of the double interaction of pesticide contamination and nestlings' sex (top) and age (bottom) on their residual baseline corticosterone. Sum of concentrations of the most prevalent pesticides were obtained by scaling substances' concentrations prior to summation. The regression lines represent the predicted values for the effect of pesticide contamination on the residuals of baseline corticosterone 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 for the top plot; and to chicks aged of approximately 15 days old (mean age – 1 SD) in green and approximately 26 days old (mean age + 1 SD) in orange for the bottom plot.

substances have been found: chlorpyrifos-methyl, picloram and acet-amiprid. Chlorpyrifos-methyl is known for its endocrine-disrupting effects in rats notably [63] and acetamiprid has been found to alter corticosterone concentrations in males house sparrow [42]. Picloram however has never been studied, to our knowledge, for its effects on birds' corticosterone although this substance is listed as having endocrine disrupting properties [63]. Nonetheless, neither maximal corticosterone nor stress-induced response in corticosterone were correlated with contamination levels. Alterations in corticosterone secretion might arise from the combination of multiple stressors and by assessing pesticide contamination levels alone we do not consider other stressors acting in concert [8]. A previous study conducted on the same population of Montagu's harriers monitored in 2018 investigated the role of anthropization on the physiological stress of fledglings and showed no relationship between baseline corticosterone and infrastructure density around the nests while maximal corticosterone was related to infrastructure density interacting with chicks' sex [79]. Thus, neither anthropization nor pesticide contamination levels alone explain the variation in physiological stress of chicks but are rather conditioned by other factors as the environment (yearly variations in food supply) or nestlings' sex. Meteorological conditions varying among years and seasons may be of great influence on the impact of pesticides on the feedback mechanisms of the HPA axis, with eventually more

pronounced alterations under stressful conditions [42]. Therefore, another factor or concurrent factors are responsible for variations in chicks' plasmatic corticosterone. Besides, the lack of effect of pesticide mixtures on stress-induced corticosterone levels may be due to a high inter-individual variability, or to the sensitivity of this endpoint to pesticide stressors, and we may have observed different responses using other physiological stress endpoints such as the ratio of heterophils to lymphocytes or reactive oxygen species production [34,41]. 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, more contaminated females were shorter-legged than expected from the general growth curve. This result may be linked to the effects of some pesticides on calcium deposition in bones [72]. Looking at the three nestlings having the highest contamination loads and delayed tarsus growth, three common pesticides (dicamba, chlorpyrifos-methyl and cypermethrin) were 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 [24]. 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 [45]. 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 damage, oxidative stress and to act as an endocrine disruptor in vertebrates [84,89,96] 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 correlated with a 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 found here) can result in behavioural changes [13,19]. 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 [88]. 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 [50]. Both *in ovo* exposure and oral ingestion of cypermethrin (alone or in mixture) reduced body weight gain in a dose-dependent manner [45,49,88]. Thus, its detection in the three nestlings with the highest pesticide load (within the 10–17 days-old period) and with poorer body condition than expected supports 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 [37], this may be the underlying link between pesticide contamination and altered body mass. For instance, in house sparrows (*Passer domesticus*), gut microbiome altered with antibiotics resulted in depressed nestling growth [48]. Therefore, an interesting prospect would be to investigate the potential effects of pesticides on the nestlings' gut microbiota. An alternative explanation that does not exclude the aforementioned ones, is the adverse effect of some substances on calcium deposition in bones via endocrine disruptions that may reduce bone density that, along with the shorter tarsus, may result in the lowered SMI of the most contaminated chicks [31,72].

The contrasting effects between younger and older nestlings for both growth and physiological stress 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 better metabolisation and excretion capacities later in their development [25]. 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, energy storage and corticosterone maintenance. Later-on, when their potential of detoxification is greater, they may have more energy to allocate for growth. 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) [28], 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, [28]). 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 [28]. 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 [56]. 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 [83] 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 [64]. However, in European shag nestlings, hepatic levels of POPs were neither associated with circulating thyroid hormones nor hepatic vitamin A despite their correlation with wing bone length [43]. Here, although higher concentrations (among the most prevalent pesticides) were correlated to delayed tarsus growth in young males, this was observed for females only when older, and altered secretions of corticosterone were found in females only. Although Montagu's harriers are sexually dimorphic, the size dimorphism is rather weak (females grow faster but are only ~19 % heavier) to explain the physiological basis behind these relationships of altered growth and corticosterone secretions and pesticide contamination. Therefore, the sex-dependent effect of pesticides could arise from endocrine disruptions altering the synthesis and metabolism of oestrogens and testosterone which ultimately interferes with higher physiological levels (see [70] for a review). The titration of steroid hormones and other biomarkers involved in their regulation could therefore be useful to investigate the origin of the differences observed among the sexes. Furthermore, 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 [43], 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 embryonic and post-hatching growth [14,51]. 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 [43]. Given the differences in the nutritional composition of the prey items, insects and passerines being poorer in energy than voles but richer in carotenoids (pigments with antioxidant properties), further investigation taking into account nestlings' diet (using food pellets for instance) are considered. Besides, one may consider that the studied nestlings are the ones that survived until fledging, therefore, more sensitive individuals may have died from the adverse effects of pesticides before their sampling. Overall, the contamination levels of chicks during their embryonic phase and food quality during their rearing period might blur the lines, allowing to observe only the surviving nestlings and counterbalancing the potential link between contamination levels and growth. The titration of contamination levels for nestlings that died before fledging would be useful just as the titration of pesticides in eggs prior to hatching. Still, to date, the egg sampling techniques would suppose the sacrifice of the embryo or at least a risk of altering its viability [74,91]. Thus, for protected species as the Montagu's harrier, only abandoned and unhatched eggs could be used to investigate the potential teratogenic effects of pesticide mixtures.

5. Conclusions

We found the first on-field evidence that cocktails of pesticides correlate to slight alterations in wild nestlings' growth and baseline

corticosterone although there is no general pattern. Younger nestlings (<17 days-old) and males (for tarsus growth) or females (for corticosterone secretion) 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. 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, one of the main limitations of our study is the small sample size which is inherent to investigations *in natura* on protected species but that challenges the statistical power of our analyses. Indeed, with a statistical power of ~70 % (see Figure S6 in supplementary materials), our models can be considered moderately robust, so that conclusions must remain cautious, especially if the effect is not detected or is marginally significant. This also implies that the reproducibility of the effect (in other populations or environments) is plausible but not guaranteed. Further investigations would be needed to prove the repeatability of the correlational relationships found here, although we expect pesticide effects to be species-specific and dependent of both the population's variability and the context, i.e., depending on the mixtures of pesticides found locally. Therefore, we cannot assert the generalization of our findings, yet our study provides pieces of knowledge into the adverse effects of pesticide cocktails on wild farmland birds *in natura*.

Environmental implication

Pesticides are considered the main factor responsible for the decline of farmland birds, as they may induce sublethal effects on non-target organisms even at low doses; however, their effects on the health of wild birds exposed to pesticide cocktails are unknown, thus we aimed at investigating the effects of pesticide mixtures on the growth and physiological stress of wild raptor's nestlings *in natura*, which is crucial to better understand the hazards pesticide may pose to wildlife, and in a *One Health* framework, to the environment and human health.

CRediT authorship contribution statement

Karine Monceau: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **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.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.139346.

Data availability

Data will be made available on request.

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